The Journal of Physiology

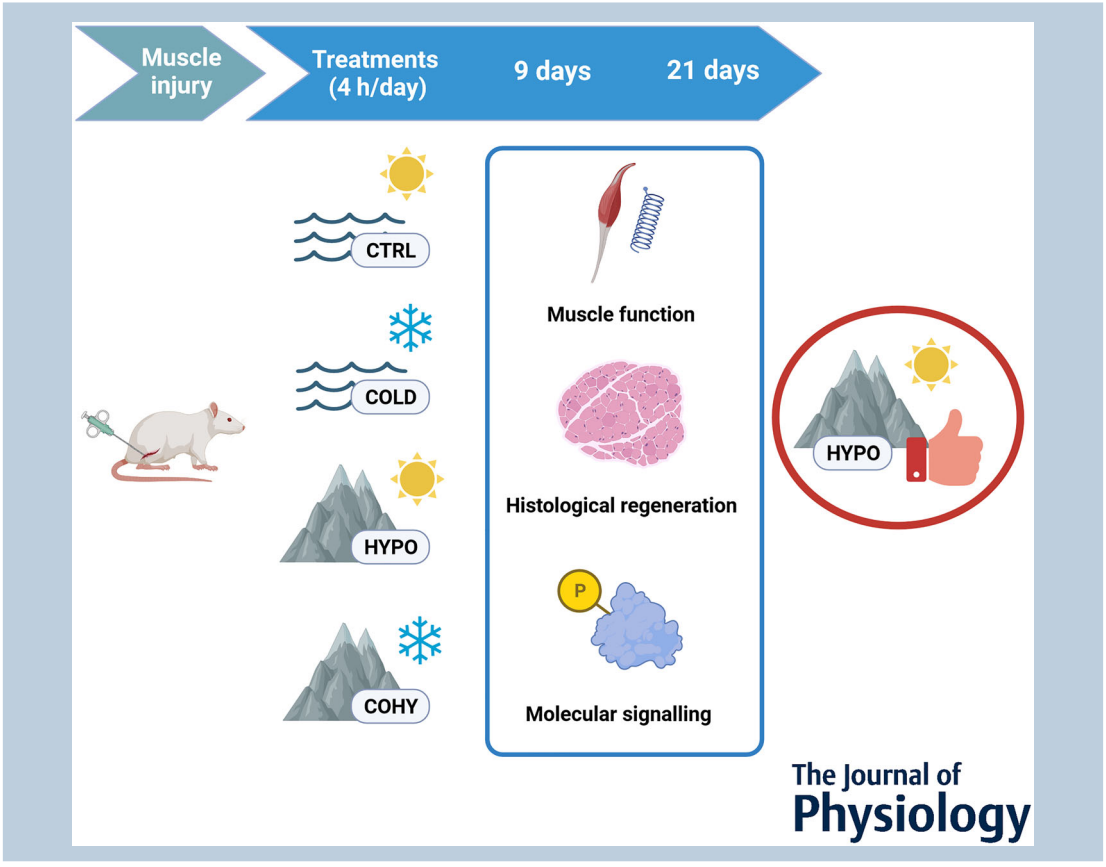
Simulated altitude is medicine: intermittent exposure to hypobaric hypoxia and cold accelerates injured skeletal muscle recovery

Garoa Santocildes, Ginés Viscor, Teresa Pagès and Joan Ramon Torrella

Secció de Fisiologia, Departament de Biologia Cel·lular, Fisiologia i Immunologia, Facultat de Biologia, Universitat de Barcelona, Barcelona, Spain

Handling Editors: Karyn Hamilton & Mike Stembridge

The peer review history is available in the Supporting information section of this article (https://doi.org/10.1113/JP285398#support-information-section).



Garoa Santocildes (Izarra-Urkabustaiz, 1992) graduated with a Bachelor's degree in Sports Sciences from the University of Zaragoza (2014) and a Master's degree in Integrative Physiology from the University of Barcelona (2016). She obtained her PhD in Biomedicine from the University of Barcelona (2021), where she is currently a postdoctoral researcher in the Department of Cellular Biology, Physiology and Immunology. Her research focuses on the effects of hypobaric hypoxia and exercise on muscle physiology and regeneration.

DOI: 10.1113/JP285398

Abstract Muscle injuries are the leading cause of sports casualties. Because of its high plasticity, skeletal muscle can respond to different stimuli to maintain and improve functionality. Intermittent hypobaric hypoxia (IHH) improves muscle oxygen delivery and utilization. Hypobaria coexists with cold in the biosphere, opening the possibility to consider the combined use of both environmental factors to achieve beneficial physiological adjustments. We studied the effects of IHH and cold exposure, separately and simultaneously, on muscle regeneration. Adult male rats were surgically injured in one gastrocnemius and randomly assigned to the following groups: (1) CTRL: passive recovery; (2) COLD: intermittently exposed to cold (4°C); (3) HYPO: submitted to IHH (4500 m); (4) COHY: exposed to intermittent simultaneous cold and hypoxia. Animals were subjected to these interventions for 4 h/day for 9 or 21 days. COLD and COHY rats showed faster muscle regeneration than CTRL, evidenced after 9 days at histological (dMHC-positive and centrally nucleated fibre reduction) and functional levels after 21 days. HYPO rats showed a full recovery from injury (at histological and functional levels) after 9 days, while COLD and COHY needed more time to induce a total functional recovery. IHH can be postulated as an anti-fibrotic treatment since it reduces collagen I deposition. The increase in the pSer473Akt/total Akt ratio observed after 9 days in COLD, HYPO and COHY, together with the increase in the pThr172AMPKα/total AMPKα ratio observed in the gastrocnemius of HYPO, provides clues to the molecular mechanisms involved in the improved muscle regeneration.

(Received 28 July 2023; accepted after revision 12 December 2023; first published online 28 December 2023)
Corresponding author G. Viscor: Secció de Fisiologia, Departament de Biologia Cel·lular, Fisiologia i Immunologia, Facultat de Biologia, Universitat de Barcelona, Av. Diagonal 643, E-08028 Barcelona, Spain. Email: gviscor@ub.edu

Abstract figure legend Effects of intermittent exposure to cold (COLD), intermittent hypobaric hypoxia (HYPO) or intermittent simultaneous exposure to both stimuli (COHY) on rat gastrocnemius muscle regeneration after 9 or 21 days of treatment. Muscle injury was surgically induced and the recovery process was assessed on muscle function (force production), histological regeneration markers and molecular signalling proteins. Overall, HYPO treatment resulted in the best intervention for muscle structure and function recovery even after only 9 days of treatment.

Key points

- Only intermittent hypobaric exposure accelerated muscle recovery as early as 9 days following injury at histological and functional levels.
- Injured muscles from animals treated with intermittent (4 h/day) cold, hypobaric hypoxia or a simultaneous combination of both stimuli regenerated histological structure and recovered muscle function 21 days after injury.
- The combination of cold and hypoxia showed a blunting effect as compared to hypoxia alone in the time course of the muscle recovery.
- The increased expression of the phosphorylated forms of Akt observed in all experimental groups could participate in the molecular cascade of events leading to a faster regeneration.
- The elevated levels of phosphorylated AMPKα in the HYPO group could play a key role in the modulation of the inflammatory response during the first steps of the muscle regeneration process.

Introduction

Muscle injuries have the highest occurrence rates in sports (Edouard et al., 2016; Hamilton et al., 2015; Järvinen et al., 2014) with an incidence of 10–55% of all reported injuries (Järvinen et al., 2007; Maffulli et al., 2014). Biarticular muscles are the most susceptible to injury, the hamstrings, abductors and gastrocnemius are the most commonly affected (Maffulli et al., 2014; Malliaropoulos et al., 2011; Mueller-Wohlfahrt et al., 2013; Valle et al., 2018).

Muscle injuries are characterized by loss of proper muscle architecture, accompanied by loss of muscle function (Forcina et al., 2020). However, upon injury, muscle tissue presents a high capacity for self-repair and for restoring its previous architecture and function. Muscle regeneration is an orchestrated homeostatic

process in which different molecular and cellular events are activated sequentially (Forcina et al., 2020). The regeneration process follows a fairly constant sequence of steps, characterized by interrelated and time-dependent phases taking place at the molecular, morphological and functional levels. These phases of the healing process can be summarized as follows (Chargé & Rudnicki, 2004; Ciciliot & Schiaffino, 2010; Dueweke et al., 2017; Forcina et al., 2020; Laumonier & Menetrey, 2016; Mittal et al., 2014; Roman et al., 2021; Toumi et al., 2006; Turner & Badylak, 2012).

1. Muscle degeneration and necrosis phase. Characterized by the necrosis of the damaged myofibres, there is an alteration of the plasmalemma permeability and therefore an uncontrolled ionic flux, a dysfunction of the organelles and a loss of muscular architecture. In addition, a haematoma is formed.
2. Inflammatory phase. There is an infiltration of inflammatory cells at the site of injury. Neutrophils are the first cells to arrive, displaying phagocytic activity and releasing pro-inflammatory cytokines, chemokines and growth factors. With the decline of neutrophils, macrophages are then the predominant inflammatory cells, initially being responsible for debris removal and secretion of pro-inflammatory cytokines, and later switching to an anti-inflammatory role and supporting myogenesis. Concomitantly, the reactive oxygen species released in the course of the 'respiratory burst' of infiltrated leukocytes also play an important role during this phase.
3. Regeneration phase. Muscle stem cells, namely satellite cells (MuSCs), are responsible for muscle reconstruction. In front of the site of injury, they are activated, proliferate and differentiate, forming the myoblasts. Myoblasts can fuse with existing myofibres, thereby repairing damaged muscle fibres, or they can fuse with each other and form new myofibres. However, other stem cells (such as endothelium-associated cells, interstitial cells and a bone marrow-derived side population), fibro-adipogenic progenitors and myonuclei are also involved in the repair of damaged tissue.
4. Tissue remodelling and maturation. In the last steps of the regenerative process, the formation and maturation of new myofibres and the repair of the damaged fibres takes place. To ensure complete restoration of the muscular system, a reconstruction of the extracellular matrix, vessels and re-innervation of the muscle fibres is also necessary.
5. Re-innervation and functional recovery. The healing process can be considered complete when the muscle fibres recover their contractile capacity and are properly innervated.

For a long time, conservative treatments, such as RICE (rest, ice, compression, elevation), massage, early mobilization and NSAIDs (non-steroidal anti-inflammatory medications), have been used to reduce pain, inflammation and swelling in the acute phase of the injury (Ciciliot & Schiaffino, 2010; Järvinen et al., 2014; Laumonier & Menetrey, 2016). However, in recent years, new therapies have increased in popularity, such as those involving ultrasound (Dueweke et al., 2017; Järvinen et al., 2014), platelet-rich plasma (Contreras-Muñoz et al., 2017; Dueweke et al., 2017), growth factors (Huard et al., 2002; Laumonier & Menetrey, 2016; Li et al., 2001), gene therapy (Huard et al., 2002), anti-fibrotic therapy (inhibitors of TGF-β1) (Huard et al., 2002; Laumonier & Menetrey, 2016) and stem cells (Contreras-Muñoz et al., 2021; Laumonier & Menetrey, 2016; Shadrin et al., 2016). Due to the high incidence of muscle injuries and their clinical importance, the muscle regeneration process has been well characterized and studied in depth. However, currently no gold standard therapy exists for enhancing the repair process, reducing the time needed to ensure total muscle regeneration at both the histological and the functional levels.

In the search for new treatments for muscle injury, hypobaric hypoxia (HH) has been postulated as an interesting tool. HH is characterized by a reduction of atmospheric oxygen partial pressure (P_{O_2}), which results in a decrease of the arterial oxygen content and therefore in tissue hypoxia (Lundby et al., 2009). The physiological differences between normobaric (reduced oxygen concentration) and hypobaric (low environmental pressure) hypoxia are controversial (Girard et al., 2012; Hauser et al., 2016; Millet et al., 2012; Mounier & Brugniaux, 2012). However, scientific evidence has accumulated on their different outcomes (Beidleman et al., 2014; Boos et al., 2016; Bourdillon et al., 2023; Coppel et al., 2015; Dipasquale, 2017; Fulco et al., 2013; Millet et al., 2013; Savourey et al., 2003). It is assumed that HH induces more intense oxidative stress than normobaric hypoxia (Burtscher et al., 2022). In consequence, oxidative stress-related phenomena could be elicited more intensely by applying this kind of hypoxia exposure. Almost all mammal cells are able to sense and respond to changes in oxygen pressure, and thus to adapt and maintain adequate oxygen levels and cell homeostasis (Semenza, 1998). Intermittent hypoxia, characterized by long periods in normoxia interspersed by periods in hypoxia, has been widely studied in the last few decades, due to its role in triggering many benefits such as increasing erythropoiesis and aerobic capacity, improving muscle capillarization and metabolism, controlling hypertension, accelerating tissue repair, improving bronchial asthma, regulating the metabolic syndrome and improving altitude acclimatization (Santocildes et al., 2021; Viscor

et al., 2018). In our laboratory, we have reported that a programme of exposure to intermittent hypobaric hypoxia (IHH) combined with aerobic exercise improves muscle recovery after the muscle damage produced by exhaustive eccentric exercise (Núñez-Espinosa et al., 2015; Rizo-Roca, et al., 2017).

Surprisingly, most previous research has studied the effects of HH in isolation, ignoring that in the biosphere HH always coexists with cold, since it is well known that temperature falls with increasing altitude at the rate of about 1°C for every 150 m (Luks et al., 2021). Natural selection could have driven the development of adaptive mechanisms in mammals to cope with both opposite stimuli because cold implies an increased oxygen requirement whereas altitude (HH) entails lower oxygen availability. Thus, the responses of an organism to simultaneous HH and cold would trigger additive or synergistic mechanisms for improving muscle irrigation that could provide the injured tissue with extra nutrients and growth factors responsible for accelerating muscle recovery. In fact, previous work from our laboratory has shown that simultaneous IHH and cold counteract the pro-inflammatory effects and weight loss produced by IHH, reduce right ventricle hypertrophy caused by hypoxia-induced pulmonary hypertension, and increase red blood cell counts and haemoglobin concentration more than HH alone (Ramos-Romero et al., 2020; Santocildes et al., 2021).

The present work aimed to investigate whether IHH could improve the muscle regeneration process after injury and if the simultaneous application of IHH and intermittent cold exposure (ICE) could trigger different responses in the time course of the regeneration process from the molecular to the histological and the functional levels.

Methods

Ethical approval

All procedures were performed in accordance with European Union guidelines for the care and management of laboratory animals and were under license from the Catalan authorities (reference no. 1899), as approved by the University of Barcelona's Ethical Committee for Animal Experimentation (reference no. 8784) for the Project entitled *Synergistic effect of cold and hypoxia in the repair of induced muscle damage in laboratory rats* funded by the Ministry of Economy and Competitiveness (Spanish Government) with code DEP2013-48334-C2-1-P. We understand the ethical principles under which the journal operates and that our work complies with this animal ethics checklist.

Animals

Eighty-six 7-week-old male Sprague-Dawley rats (Envigo RMS, Barcelona, Spain), with an initial weight of 211 ± 28 g were used in the study. Rats were housed at 23 ± 2°C and maintained on a 12 h light–dark cycle, with *ad libitum* access to water and food during the experiments (including during cold and hypoxia exposures).

After 1 week of quarantine, rats were anaesthetized with inhaled 2–3.5% isoflurane (IsoFLo, Zoetis, Louvain-La-Neuve, Belgium) in 100% oxygen at 1 L/min (maintenance dose 1.5–3.5%) and skeletal muscle injury was surgically induced on all right gastrocnemius (GAS) according to a previously described method (Contreras-Muñoz et al., 2016). Briefly, a traumatic muscle lesion was generated by using an 18 G biopsy needle with a 0.84 mm inner diameter (Bard Monopty Disposable Core Biopsy Instrument, Bard Biopsy Systems, Covington GA, USA) by performing a transversal biopsy in the leg medial GAS muscle (7 mm from the end of the Achilles tendon and 2 mm depth). It has been demonstrated by magnetic resonance imaging (MRI) that this surgically induced muscle injury closely mimics the anatomical characteristic of sports injuries (grade I–II), and also the complete histological and functional processes of degeneration–regeneration that are observed after human sports injuries (Contreras-Muñoz et al., 2016). A post-surgical single dose of analgesia (buprenorphine 0.01 mg/kg) was subcutaneously administered to all intervention rats immediately after surgery (Contreras-Muñoz et al., 2016).

After induction of skeletal muscle injury, all animals were randomly divided into five groups: (1) Control (CTRL): animals that recovered passively from the injury in normoxia at 23°C; (2) Intermittent cold (COLD): animals enrolled in an ICE protocol in a cold room at 4°C for 4 h per day; (3) Intermittent hypobaric hypoxia (HYPO): animals exposed to a simulated altitude for 4 h per day by using a hypobaric chamber with an internal pressure of 577 ± 3 hPa simulating 4500 m of geographical altitude, and thus implying P_{IO_2}≈80 mmHg, equivalent to 11.5% oxygen concentration at sea level, almost a half (55%) of the sea level oxygen availability (Santocildes et al., 2021); and (4) Intermittent cold and hypobaric hypoxia (COHY): animals simultaneously submitted to hypobaric hypoxia (4500 m) and cold (4°C) for 4 h per day. Animals from these different groups were submitted to the assigned treatment for 9 or 21 days, starting the day after the injury procedure. An extra group (CTRL_0) was considered to verify the procedure of surgical injury and the *in vivo* muscle functional procedures used to assess the muscle recovery process. This fifth group of animals was injured and, after 24 h, they were assessed for the functional muscle force test detailed below.

In vivo muscle force measurements

In vivo determination of GAS muscle contractile capabilities was carried out the day after the last treatment session. Animals were anaesthetized by an intraperitoneal injection consisting of a mixture of ketamine (75 mg/kg) and xylazine (10 mg/kg). Before beginning the surgical protocol, assurance that the animals were under deep anaesthesia was tested by assessing the absence of both corneal and pedal withdrawal reflexes. If a positive response for any of those reflexes was detected, a 10% additional anaesthetic dose was administered until deep anaesthesia was reached. Thereafter, animals were placed in the prone position on a dissection platform, with the limb immobilized with two needles (one in the knee and one in the ankle) with the knee in a fully extended position. The distal end of the calcaneal tendon was exposed and cut at the insertion of the calcaneus bone, and the GAS muscle was then separated from the surrounding tissue, leaving the proximal insertion, blood supply and nerves intact. The common peroneal nerve was cut, to avoid recruitment of the ankle dorsal flexors, and therefore significant reduction of torque. Once the GAS muscle was completely isolated, the calcaneal tendon was tied to a force transducer (MLT 1030/D, ADInstruments, Colorado Springs, CO, USA) through a thread and stretched with an initial passive force of 30 mN. Through a lateral incision on the thigh, the sciatic nerve was exposed and directly stimulated by an electrode connected to an electrical stimulator (Stimulus Isolator, FE180, ADInstruments). All measurements were made using PowerLab/16SP data acquisition hardware (ADInstruments) and analysed using LabChart Version 7 Sofware (ADInstruments). To ensure optimal contraction conditions, exposed muscles were covered with mineral oil (Sigma-Aldrich, St Louis, MO, USA) to prevent muscle drying during the intervention. Moreover, muscle temperature was checked using a non-contact infrared thermometer (PCE FIT-10) and maintained in a narrow range of variation (32–35°C) through a heat lamp (Daylight Basking Spot lamp 50 W; Exo Terra, Montreal, Canada).

To set up a supramaximal response of the muscle, the pulse width was set at 0.05 ms (following the manufacturer's specifications) and the maximal twitch response was determined by increasing the intensity of the stimulus current, starting at 0.1 mA, until further increases in stimulator intensity did not produce a further increase in twitch amplitude. Thus, to ensure a supramaximal stimulation of the muscle, the intensity which produced the maximal response was multiplied by a factor of 1.5, resulting in 3 mA.

In each GAS muscle, the optimal contraction length (L_0) in which maximal twitch force is produced was checked before carrying out the force tests. Then, the following parameters were obtained to assess the functional capacity of the muscle.

1. Twitch peak force (PF, in mN). The muscle force produced after an isolated stimulus. It was obtained as the average of five consecutive twitches at a frequency of 1 Hz. Twitch records were also used to analyse the contraction time (CT, in ms) and the half-relaxation time (HRT, in ms).
2. Maximum tetanic force (TetF). Trains of stimuli were given for 1 s starting at a frequency of 10 Hz and increasing the stimulation frequency by 10 Hz in each train of stimuli up to TetF. Pauses of 1 min between trains were allowed to avoid muscle fatigue (Allen et al., 2008).
3. Low-frequency fatigue (LFF). At the end of the TetF protocol, 5 min of recovery was allowed before developing the fatigue protocol, which was performed by continuous muscle stimulation for 2 min at a frequency of 30 Hz. A fatigue index was obtained by measuring the area between the force record and the baseline (force–time fatigue, in N·s).

For each animal, all measurements were performed in the GAS muscle of both legs, considering the non-injured leg as a control (Santocildes et al., 2022). All obtained records were normalized to muscle weight.

Once muscle force measurements were completed, GAS muscles were excised, weighed, frozen in pre-cooled 2-methylbutane (Sigma-Aldrich) and stored at −80°C until further analysis. After ending the procedure, animals were killed via an intraperitoneal injection of sodium pentobarbital (150 mg/kg) following the AVMA updated version of the *Guidelines for the euthanasia of Animals* (American Veterinary Medical Association, 2020).

Protein extraction and immunoblotting

For protein analysis, whole GAS muscles were homogenized in urea lysis buffer (6 M urea, 1% SDS), supplemented with protease and phosphatase inhibitors (Complete Protease Inhibitor Cocktail and PhosphoSTOP, Sigma-Aldrich) using the Precellys Evolution tissue homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France). The lysates were centrifuged at 25,000 *g* for 15 min at 4°C to remove cell debris and the total protein content of the collected supernatants was quantified by the bicinchoninic acid assay (Thermo Fisher Scientific, Waltham, MA, USA). Muscle protein extracts were solubilized in an electrophoresis loading buffer (62.5 mM Tris/HCl, pH 6.8; 2.3% SDS; 10% glycerol; 5% 2-mercaptoethanol and bromophenol blue) and equal amounts of protein were loaded onto each lane

of the gel and electrophoresis was run on SDS-PAGE gels.

After electrophoresis, proteins were transferred to an Immuno-Blot PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA) for protein blotting. To verify that equal amounts of muscle protein were charged in each well and the efficiency of the transference, membranes were stained with Ponceau stain (Sigma-Aldrich). For immunoblotting, membranes were blocked in 4% BSA in TBS-T (Tris-buffered saline containing 0.1% Tween 20) for 1 h at room temperature. To detect our target proteins, membranes were incubated overnight at 4°C with primary antibodies against phosphorylated forms pSer2448mTor, pThr172AMPKα and pSer473Akt (Cat. no. 2971, RRID: AB_330 970; Cat. no. 2535, RRID: AB_331 250; Cat. no. 4060, RRID: AB_2 315 049, Cell Signaling Technology, Danvers, MA, USA) diluted 1:500 in 4% BSA-TBS-T. Subsequently, the membranes were incubated with the corresponding secondary HRP-conjugated antibody (Cat. no. 31 460, RRID: AB_228 341; Cat. no. 31 430, RRID: AB_228 307, Thermo Fisher Scientific) diluted 1:5000 in 5% Blotto in TBS-T for 1 h at room temperature. The specific bands were visualized with the clarity TM Western ECL Substrate Kit (Bio-Rad Laboratories) and the chemiluminescence signal was measured using the Odyssey Fc Imaging System (LI-COR Inc. Biotechnology, Lincoln, NE, USA) and quantified with the Image Studio Software (v. 5.2.5, LI-COR Inc. Biotechnology). Membranes were then stripped off using a stripping buffer (Tris/HCl 1 M pH 6.7, SDS 20%, 2-mercaptoethanol 14.3 M). Then, membranes were again re-stained with Ponceau stain, re-blocked and re-incubated with secondary HRP antibody to check the efficiency of the stripping. Subsequently, membranes were re-blocked and incubated with the corresponding antibody of the total form of the protein mTOR, AMPKα and Akt (diluted in 1:1000 in 4% BSA-TBS-T) (Cat. no. 2983, RRID: AB_2 105 622; Cat. no. 5831, RRID: AB_10 622 186; Cat. no. 9272, RRID: AB_329 827, Cell Signaling Technology).

For protein expression analysis, an aliquot of the same control sample (rat tibialis anterior muscle lysate, prepared as per the experimental samples) was loaded in triplicate on all gels. This allowed us to compare the samples of the different experimental groups that were loaded on different membranes. In every membrane, each band, including the control samples, was normalized to the Ponceau stain and then again to the mean of the three control samples from the corresponding membrane. Finally, protein expression data, expressed as arbitrary units (AU), were reported and these values were statistically analysed to compare the different experimental groups.

Muscle histology

Histochemistry: fibre type and size. Frozen GAS muscles were embedded in a mounting medium (Tissue-Tek O.C.T. Compound, Sakura Finetek, Toorrance, CA, USA) and cut in serial transverse sections (14–16 μm) using a cryostat (CM3050S, Leica, Wetzlar, Germany) at −22°C. Sections were mounted on gelatinized glass slides (0.02%) and then incubated for 5 min in a buffering fixative (Viscor et al., 1992) to prevent shrinkage or wrinkling. For histochemical analysis, muscle sections were stained for myofibrillar adenosine triphosphatase (mATPase), following pre-incubation in an alkaline solution (pH 10.7), to differentiate between slow-twitch (Type I) and fast-twitch (Type II) fibres and to measure fibre cross-sectional area (FCSA). Photomicrographs of stained muscle sections were obtained with a light microscope (BX61; Olympus, Tokyo, Japan) connected to a digital camera (DP70, Olympus). Images were taken at 20× magnification and were analysed using ImageJ software (v. 1.51n, National Institutes of Health, Bethesda, MD, USA).

Histology and immunofluorescence: analysis of muscle injury and regeneration. Consecutive cross-sections of each injured muscle were cut (12 μm) using a cryostat (Leica CM3050S) at −22°C, starting at the myotendinous junction of the GAS muscle until the end of the injured area. For histological analysis, slides were stained with haematoxylin–eosin. For that, muscle sections were placed for 2 min in haematoxylin solution (Harrys haematoxylin, Sigma-Aldrich) and 1 min in 1% eosin (Eosin, Sigma-Aldrich), sections were then dehydrated in graded ethanol solutions for 1 min in each solution (ethanol 50%, ethanol 70% ×2, ethanol 90%, ethanol 100% ×2) and finally cleared in xylene (5 s) and mounted with DPX medium (Sigma-Aldrich). For immunofluorescence, muscle sections were fixed in cold acetone for 10 min, air-dried and blocked with 3% BSA in PBS (phosphate-buffered saline) for 10 min at room temperature. Then, muscle samples were incubated for 18 h in a humid chamber at 4°C with primary antibodies against Developmental Myosin Heavy Chain (dMCH) (Cat. no. NCL-MHCd, RRID: AB_563 901, Leica Biosystems) and Collagen I (Cat. no. ab34710, RRID: AB_731 684, Abcam, Cambridge, MA, USA) diluted 1:100 in 3% BSA-PBS. After that, samples were incubated for 1 h in a dark humid chamber with secondary antibodies Alexa Fluor 488 anti-rabbit and Alexa Fluor 568 anti-mouse (Cat. no. A32731, RRID: AB_2 633 280; Cat. no. A-11 004, RRID: AB_2 534 072, Thermo Fisher Scientific) diluted 1:1000 in 3% BSA-PBS. Finally, slides were mounted with DAPI (Fluoromount-G containing

DAPI medium, Sigma-Aldrich). Images were acquired with a fluorescence microscope (DFC360 FX, Leica) at 10× magnification and were analysed by ImageJ software (v.1.51n, National Institutes of Health, USA). Obtained images were used to analyse the total number of dMHC-positive fibres and their FCSA and the localization of fibre nuclei. Moreover, the total amount of Collagen I deposited at the injury site was quantified.

Statistical analysis

We applied a two-way repeated-measures ANOVA considering the injured and their contralateral intact muscle from the same animal as a paired factor, and time of sampling (9 or 21 days) and treatment (CTRL, HYPO, COLD, COHY) as independent factors. Normality tests (Shapiro-Wilk), and equal variance tests (Brown-Forsythe) were performed. *Post hoc* all pairwise multiple comparison procedures (Bonferroni *t* test) allowed us to study interactions within and between independent factors. Figures are presented as box-and-whisker plots or as histograms with individual subject data points plotted. In the box-and-whisker plots, the box represents the interquartile range and shows the first and the third quartiles separated by the median. Whisker endpoints represent the minimum and maximum values, and the mean is indicated in the box with a cross. In the histograms, tables and text data summaries, data are presented with the mean and the standard deviation (SD) as a dispersion parameter. The exact *P*-values are stated throughout the text, figures and tables considering $P < 0.05$ as statistically significant. All statistical analysis was performed using the statistical package SigmaPlot 14 (Systat Sofware, Inc., 2017, Chicago, IL, USA) and figures were designed using Graph Pad Prism 9 (Graph Pad Sofware, Inc., 2020, La Jolla, CA, USA).

Results

Muscle injury procedure

Animals from the CTRL_0 group showed a significant decrease in PF, TetF and LFF 24 h after the surgical muscle injury ($P = 0.0012$, $P = 0.0008$ and $P = 0.007$, respectively) (Fig. 1). However, no changes were observed between the injured and uninjured GAS muscle in CT (39.2 ± 5.5 *vs.* 39.0 ± 3.5 ms, $P = 0.922$) and HRT (67.7 ± 12.6 *vs.* 69.0 ± 6.6 ms, $P = 0.637$) (data not shown in the graph). The injured GAS showed a significantly higher GAS/body mass ratio than the non-injured contralateral GAS (7.35 ± 0.50 *vs.* 6.92 ± 0.27%, $P = 0.007$) (data not shown). Figure 2 shows an injured area of a representative GAS muscle. The image was taken 24 h after injury when the degeneration and necrosis of the myofibres has begun. In the injured area, a disruption of the normal architecture of the muscle and the infiltration of inflammatory cells is evident.

Muscle contractile properties

To assess the time course evolution of the muscle injury and the effect of the different treatments on muscle function recovery, an *in vivo* comparison of the contractile properties of the right (injured) and left (non-injured) GAS muscle was carried out (Fig. 3). Injured GAS in CTRL, which was passively recovered from the muscle injury, showed a significant reduction in the ability to generate force both after 9 days (PF $P = 0.002$ and TetF $P < 0.001$) and after 21 days (PF $P = 0.006$ and TetF $P = 0.002$) (Fig. 3*A*–*D*). Response to fatigue (LFF) was also compromised at both 9 ($P = 0.069$) and 21 days ($P < 0.001$) (Fig. 3*E* and *F*). However, no significant changes were observed between the injured and uninjured GAS in CTRL for the twitch time-dependent parameters studied: CT at 9 days (38 ± 12 *vs.* 39 ± 10 ms, $P = 0.247$)

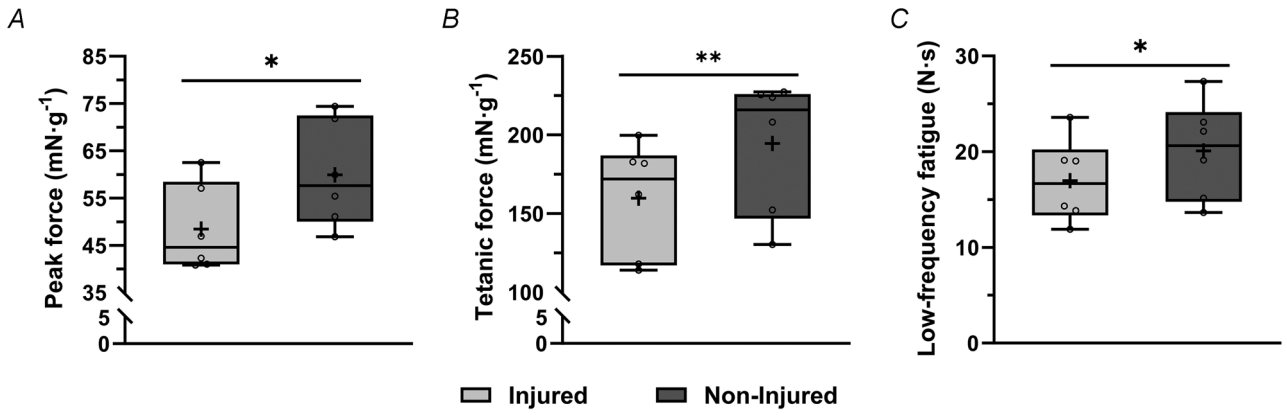


Figure 1. Gastrocnemius muscle force parameters after 24 h of injury
A, peak force; *B*, tetanic force; and *C*, low-frequency fatigue index. Statistically significant differences are indicated as: (*A*) *$P = 0.001$, (*B*) **$P < 0.001$, (*C*) *$P = 0.07$.; $n = 6$ per group. Points represent the values of each sample.

and at 21 days (44 ± 6 *vs*. 46 ± 8 ms; $P = 0.497$); and HRT at 9 days (64 ± 15 *vs*. 68 ± 16 ms; $P = 0.460$) and at 21 days (73 ± 12 *vs*. 77 ± 13 ms; $P = 0.630$) (data not shown).

Similarly, the COLD group showed after 9 days of injury deficiencies in the contractile properties in injured GAS when compared to non-injured GAS in all studied parameters (PF $P = 0.032$, TetF $P = 0.047$, LFF $P = 0.033$, CT $P = 0.012$, HRT $P = 0.046$) (Fig. 3*A*, *C* and *E*). However, after 21 days of intermittent cold treatment, the injured muscles presented no significant reductions in contractile properties (PF $P = 0.055$, TetF $P = 0.265$, LFF $P = 0.769$, CT $P = 0.954$, HRT $P = 0.630$) (Fig. 3*B*, *D* and *F*).

After 9 days of treatment, the HYPO animals did not show any significant difference between the injured and non-injured muscles in PF ($P = 0.813$), TetF ($P = 0.097$), LFF ($P = 0.452$), CT ($P = 0.892$) and HRT ($P = 0.460$) (Fig. 3*A*, *C* and *E*). In the results obtained 21 days following injury, a complete recovery of the muscular contractile function was observed as is deduced from the similar values recorded in all the parameters in both legs (Fig. 3*B*, *D* and *F*).

After 9 days of treatment, the COHY group did not recover muscle force production of the injured leg (PF $P = 0.004$, TetF $P = 0.037$) (Fig. 3*A* and *C*) but showed similar values for both legs in the resistance to fatigue at low frequency (LFF $P = 0.346$) (Fig. 3*E*) and in the time-dependent parameters (CT $P = 0.165$, HRT $P = 0.460$). However, exposure to simultaneous cold and hypoxia for 21 days recovered the capacity to generate force of the injured muscles (PF $P = 0.183$, TetF $P = 0.477$) (Fig. 3*B* and *D*).

To determine if the different treatments affected muscle strength *per se*, the ability to generate force of the non-injured muscles was compared between the different experimental groups, and non-significant differences were found in all of the parameters (Fig. 3). On the other hand, when injured muscles of the different groups were compared, statistically significant differences were found at both 9 and 21 days. At 9 days, HYPO showed significantly higher values than CTRL and COLD in PF ($P = 0.043$ *vs*. CTRL and $P = 0.037$ *vs*. COLD). Moreover, at 21 days, significant differences were observed between CTRL and the other experimental groups in TetF ($P = 0.015$ *vs*. COLD, $P = 0.002$ *vs*. HYPO and $P < 0.001$ *vs*. COHY) and in LFF ($P = 0.028$ *vs*. COLD, $P = 0.001$ *vs*. HYPO and $P = 0.010$ *vs*. COHY).

Finally, Table 1 shows that the GAS mass to body mass ratio of the injured and non-injured muscles had no significant differences between any experimental group either after 9 days or after 21 days of injury.

Distribution of the different fibre types and fibre areas

Figure 4 shows the fibre proportions, FCSA and relative distribution frequencies of the muscle fibres classified according to their contractile velocity (slow or type I and

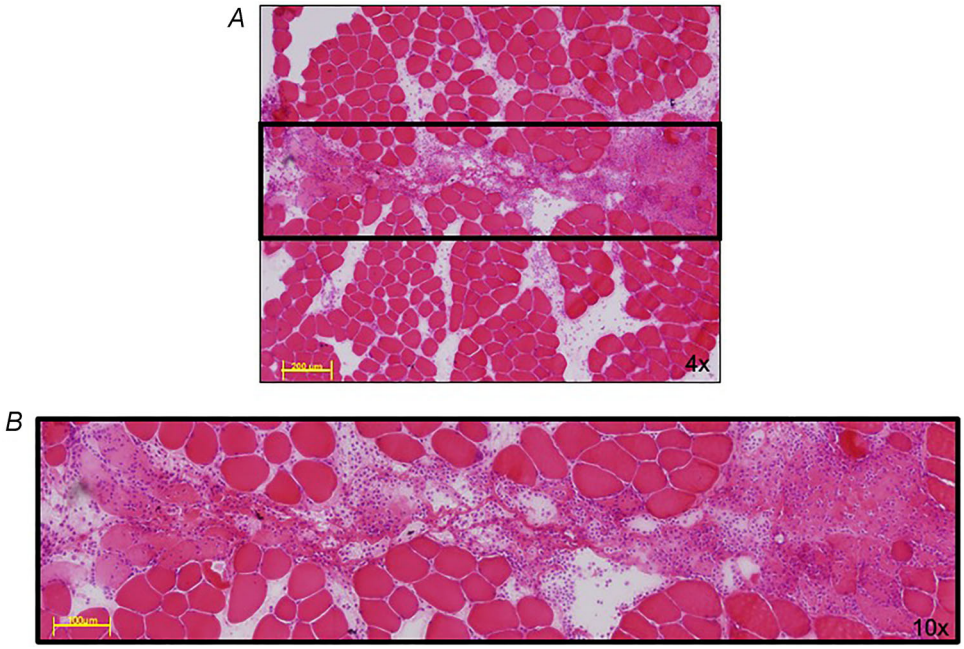


Figure 2. Representative images of haematoxylin–eosin stain of gastrocnemius muscle after 24 h of injury
A, image at 4× magnification (scale bar: 200 μm); black square indicates the muscle zone enlarged in *B* at 10× magnification (scale bar: 100 μm).

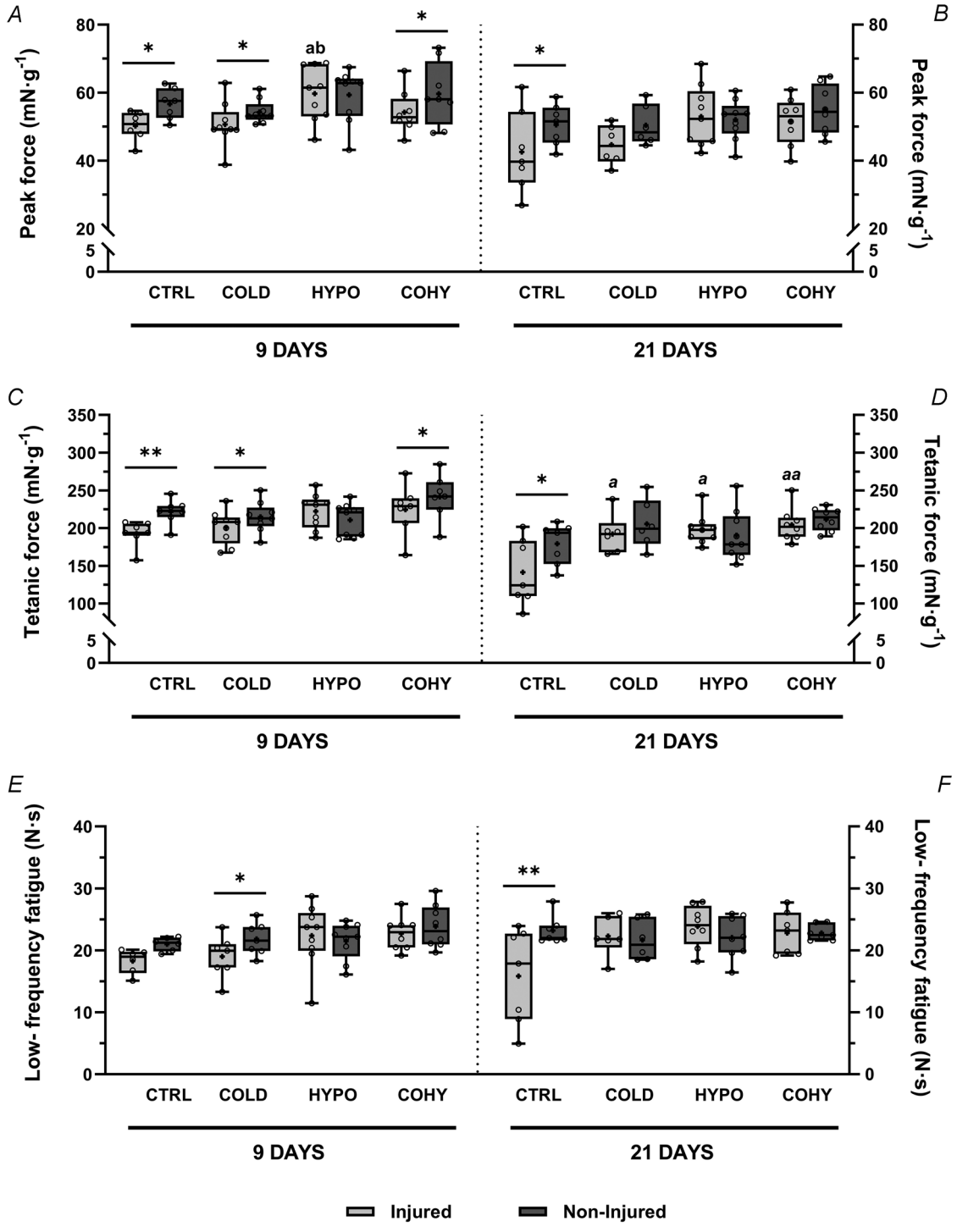


Figure 3. Measurements of gastrocnemius muscle contractile parameters after 9 and 21 days of muscle injury
A and *B*, peak force; *C* and *D*, tetanic force; and *E* and *F*, low-frequency fatigue. Statistically significant differences are indicated as: *A*, *Injured *vs.* Non-Injured, CTRL: $P = 0.002$, COLD: $P = 0.032$ and COHY: $P = 0.004$. [a]HYPO *vs.* CTRL: $P = 0.043$, [b]HYPO *vs.* COLD: $P = 0.043$. *B*, *Injured *vs.* Non-Injured, CTRL: $P = 0.006$. *C*, *Injured *vs.* Non-Injured (CTRL: $P < 0.001$, COLD: $P = 0.047$ and COHY: $P = 0.037$). *D*, *Injured *vs.* Non-Injured: CTRL: $P = 0.002$. [a]COLD *vs.* CTRL: $P = 0.015$, [a]HYPO *vs.* CTRL: $P = 0.002$, [a]COHY *vs.* CTRL: $P < 0.001$. *E*, *Injured *vs.* Non-Injured, COLD: $P = 0.033$. *F*, *CTRL: $P < 0.001$. n = 6–9 per group at each time point. Points represent the values of each sample. CTRL, control; COLD, intermittent cold; HYPO, intermittent hypobaric hypoxia; COHY, simultaneous intermittent cold and hypobaric hypoxia.

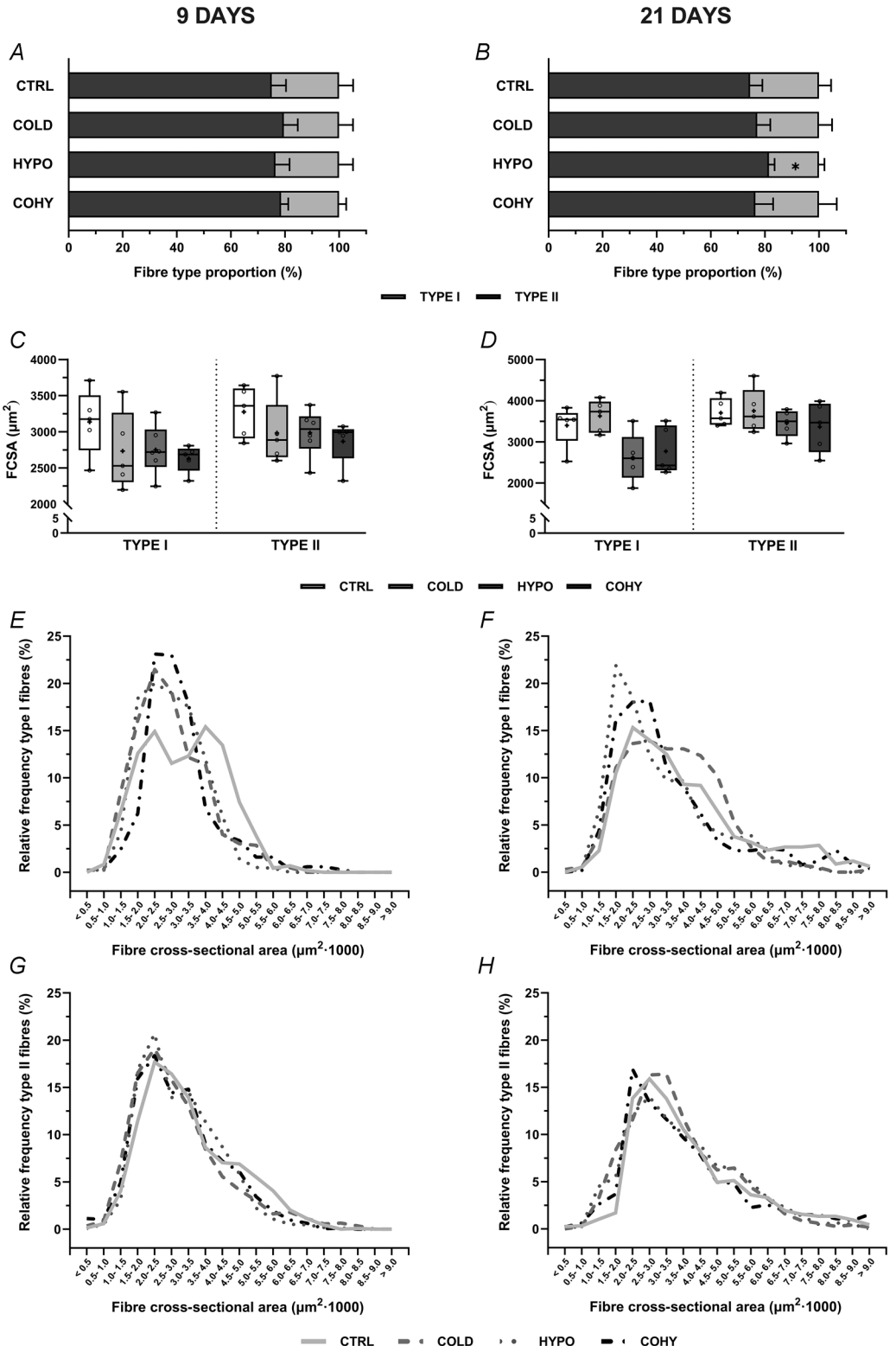


Figure 4. Fibre type proportion (*A* and *B*), fibre cross-sectional area (FCSA) (*C* and *D*) and fibre type distribution curves (*E–H*) in the gastrocnemius muscle after different treatments
Statistically significant differences are indicated as: *P = 0.032 *vs*. CTRL; n = 5–6 per group at each time point. CTRL, control; COLD, intermittent cold; HYPO, intermittent hypobaric hypoxia; COHY, simultaneous intermittent cold and hypobaric hypoxia.

Table 1. Mass of gastrocnemius muscles of the different experimental groups

		Gastrocnemius/body mass (‰)		
		Injured	Non-injured	*P*-value
9 Days	CTRL	6.70 ± 0.31	6.54 ± 0.24	0.352
	COLD	6.67 ± 0.50	6.60 ± 0.51	0.602
	HYPO	6.92 ± 0.33	6.86 ± 0.31	0.201
	COHY	6.84 ± 0.36	6.71 ± 0.35	0.190
21 Days	CTRL	7.02 ± 0.33	6.92 ± 0.48	0.488
	COLD	6.66 ± 0.25	6.34 ±0.34	0.096
	HYPO	7.11 ± 0.33	6.90 ± 0.31	0.061
	COHY	6.61 ± 0.46	6.59 ± 0.31	0.899

Data are presented as mean ± standard deviation; n = 6 to 9 per group at each time point. CTRL, control; COLD, intermittent cold; HYPO, intermittent hypobaric hypoxia; COHY, simultaneous intermittent cold and hypobaric hypoxia.

fast or type II). No significant differences were observed between groups in the fibre type proportion at 9 days (Fig. 4*A*), while at 21 days of intervention, HYPO showed a significant reduction of type I fibres ($P = 0.032$) (Fig. 4*B*). Regarding FCSA, both fibre types after 9 days of injury in all experimental groups showed a non-significant trend towards smaller fibres (Fig. 4*C*). This trend was also evident after 21 days, except for COLD which maintained the same fibre size as CTRL (Fig. 4*D*).

Figure 4*E*–*H* shows the distribution frequency curves for each fibre type based on its FCSA. After 9 days of injury, we observed that despite the absence of significant differences in type I fibres, CTRL showed a bimodal frequency curve slightly shifted to the right (Fig. 4*E*), while the frequency curves are almost superposed in type II fibres (Fig. 4*G*). After 21 days, in agreement with what was observed in the FCSA values, CTRL and COLD showed flattened type I fibre frequency curves that were slightly shifted to the right, indicating the presence of a greater number of larger fibres than HYPO and COHY (Fig. 4*F*). Type II fibres presented very similar frequency curves in all groups, indicating fibres of very similar size (Fig. 4*H*).

As can be seen in Fig. 5*A*, during the muscle regeneration process muscles from all groups presented at the injury site fibres with positive dMHC presence (red). Specifically, after 9 days of injury, ∼51% of the muscle fibres in CTRL muscles were dMHC-positive, while this number decreased up to 15% on day 21 following injury (Fig. 5*B*). The two cold-treated groups (COLD and COHY) showed 50% fewer dMHC-positive fibres than CTRL after 9 days of injury, although these differences were not statistically significant ($P = 0.104$ and $P = 0.081$, respectively). However, 21 days after injury COLD and COHY presented very similar values to CTRL, with ∼15% of dMHC-positive fibres (Fig. 5*B*). Finally, HYPO showed less than 15% of the total fibres in the injured zone reacting positively for dMHC at both 9 and 21 days. Thus, HYPO showed a significant decrease in dMHC-positive fibres after 9 days compared to CTRL ($P = 0.004$), while after 21 days the percentage of dMHC-positive fibres found at the injury site was similar in all groups (Fig. 5*B*).

Regarding the FCSA of dMHC-positive fibres (Fig. 5*C*), HYPO presented smaller fibres than the rest of the groups after 9 days, although these differences were only significant when comparing HYPO *versus* COLD ($P = 0.050$). Conversely, 21 days after injury no significant differences in size were observed between any of the groups.

At 9 days, all treated groups presented a lower percentage of fibres with central nuclei than CTRL, with these differences being statistically significant ($P = 0.006$) when comparing HYPO (29%) *versus* CTRL (45%) (Fig. 5*D*). However, 21 days after injury CTRL, HYPO and COHY had similar percentages of fibres with central nuclei (∼35%), while COLD showed a significantly higher percentage of central nuclei in the injured area ($P = 0.027$ *vs*. CTRL, $P = 0.028$ *vs*. HYPO and $P = 0.015$ *vs*. COHY) (Fig. 5*D*).

Regarding collagen I (Fig. 5*E*), the groups exposed to intermittent hypoxia (HYPO and COHY) showed less deposition in the injured area than CTRL and COLD after 9 days of injury. By contrast, after 21 days of treatment, HYPO presented lower collagen I deposition than CTRL ($P = 0.126$), COLD ($P = 0.072$) and COHY ($P = 0.032$).

Molecular responses

Akt. After 9 days of post-injury treatment, COHY presented a significant increase as compared to CTRL in the expression of the phosphorylated form of Akt (pSer473Akt) in the injured muscle ($P = 0.027$) and in the non-injured muscle ($P = 0.009$), while HYPO presented only differences when compared to CTRL in the non-injured muscle ($P = 0.046$) (Fig. 6*C*). Significant increases in comparison to CTRL in the pSer473Akt to total Akt ratio were observed in groups submitted to hypoxia (Injured muscle: HYPO: $P = 0.015$ and COHY: $P = 0.024$; Non-injured muscle: COHY: $P = 0.025$) (Fig. 6*E*). Conversely, after 21 days of post-injury treatment, no significant differences were found between any treated group and CTRL (Fig. 6*B*, *D* and *E*), and only a significant decrease in Akt expression in HYPO compared to COLD was evident in the non-injured muscle ($P = 0.011$) (Fig. 6*B*).

mTOR. Mammalian Target of Rapamycin (mTOR) after 9 days of post-injury treatment showed a significant reduction in injured muscles in those groups treated with intermittent cold (COLD: $P = 0.004$ and COHY:

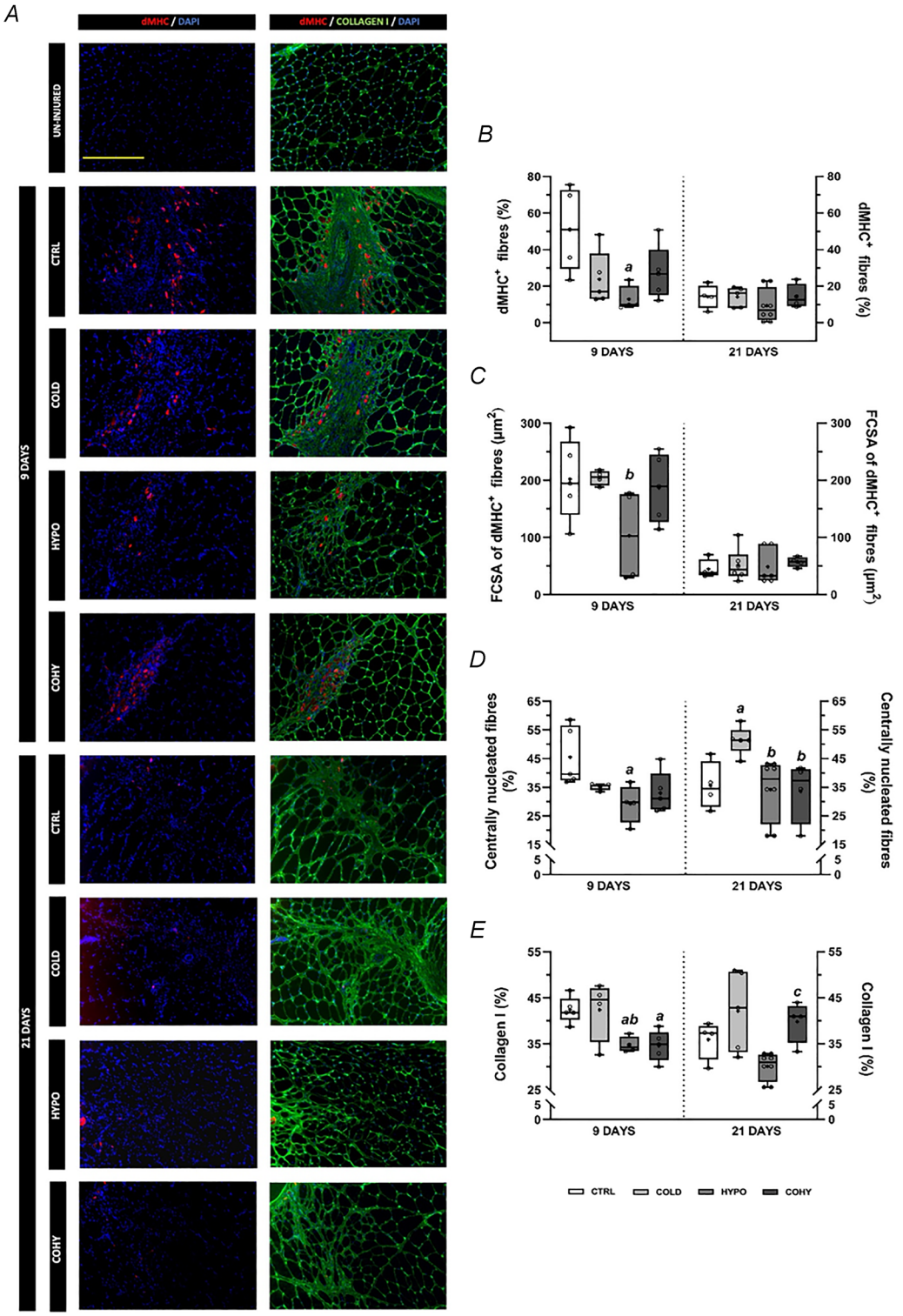


Figure 5. Histological time course of muscle regeneration
A, representative images of immunofluorescence stains; *B*, percentage of positive fibres in dMHC (developmental myosin heavy chain); *C*, fibre cross-sectional area (FCSA) of positive fibres in dMHC; *D*, percentage of fibres with central nuclei; and *E*, percentage of collagen I deposition at the injury site. Statistically significant differences are indicated as: *B*, [a]HYPO *vs.* CTRL: $P = 0.004$. *C*, [b]HYPO *vs.* COLD: $P = 0.05$. *D*, 9 days: [a]HYPO *vs.* CTRL: $P = 0.006$; 21 days: [a]COLD *vs.* CTRL: $P = 0.027$, [b]HYPO *vs.* COLD: $P = 0.028$ and COHY *vs.* COLD: $P = 0.015$. *F*, 9 days: [a]HYPO *vs.* CTRL: $P = 0.012$ and COHY *vs.* CTRL: $P = 0.007$, [b]HYPO *vs.* COLD: $P = 0.017$; 21 days: [c]COHY *vs.* HYPO: $P = 0.032$. Scale bar: 100 μm; n = 4–6 per group at each time point. Points represent the values of each sample.

CTRL, control; COLD, intermittent cold; HYPO, intermittent hypobaric hypoxia; COHY, simultaneous intermittent cold and hypobaric hypoxia.

$P = 0.007$) (Fig. 7*A*). The mTOR phosphorylated form (pSer2448mTOR) had a significantly increased expression in HYPO injured muscles ($P = 0.041$ *vs.* COLD and $P = 0.039$ *vs.* COHY) (Fig. 7*C*), while no significant changes in the pSer2448mTOR to total mTOR ratio was observed throughout the different groups (Fig. 7*F*). After 21 days of post-injury treatment, HYPO muscles had the highest total mTOR expression, being significant in non-injured GAS ($P = 0.035$ *vs.* COLD and $P = 0.039$ *vs.* COHY) (Fig. 7*B*). However, no statistical differences were found in the phosphorylated form of mTOR (Fig. 7*D*). The pSer2448mTOR to total mTOR ratio had significantly higher values in COLD non-injured muscles ($P = 0.030$ *vs.* CTRL; $P < 0.001$ *vs.* HYPO and $P = 0.019$ *vs.* COHY) (Fig. 7*E*). Finally, when injured and non-injured muscles were compared, a statistically significant increase of mTOR protein was observed in injured muscle 9 days after muscle injury ($P = 0.033$).

AMPK. AMP-activated protein kinase (AMPKα) expression after 9 days of post-injury treatment showed a significant increase in non-injured GAS in COLD ($P = 0.002$ *vs.* HYPO and $P = 0.041$ *vs.* COHY) and a significant decrease in injured muscle of HYPO when compared to COLD ($P = 0.007$). There was an increase of pThr172AMPKα and pThr172AMPKα to total AMPKα ratio in HYPO injured muscles, which was only statistically significant in pThr172AMPKα compared to COHY ($P = 0.041$), probably due to the wide dispersion of the values and the low sample size (Fig. 8*C*). After 21 days of post-injury treatment, the hypoxia-treated groups (HYPO and COHY) showed a significantly lower expression of AMPKα in injured muscle compared to COLD ($P = 0.006$ and $P = 0.022$, respectively). The most relevant finding after 21 days of post-injury treatment was the highly significant values of HYPO in pThr172AMPKα expression (Injured muscle: CTRL: $P < 0.001$; COLD: $P < 0.001$ and COHY: $P < 0.001$; Non-injured muscle: CTRL: $P = 0.001$; COLD: $P = 0.003$ and COHY: $P = 0.003$) and the pThr172AMPKα to total AMPKα ratio (Injured muscle: CTRL: $P < 0.001$; COLD: $P < 0.001$ and COHY: $P < 0.001$; Non-injured muscle: CTRL: $P = 0.030$; COLD: $P = 0.018$) (Fig. 8*F*). Moreover, significant differences in HYPO were found between injured and contralateral non-injured muscles in pThr172AMPKα at 9 days ($P = 0.007$) (Fig. 8*C*) and in pThr172AMPKα to total AMPKα ratio at 21 days ($P = 0.002$) (Fig. 8*F*). Representative images of blots for all analysed proteins are depicted in Fig. 9.

Discussion

The present study aimed to establish the potential role of intermittent exposure to cold and hypobaric hypoxia on regenerative medicine. Here we report the effect of ICE and IHH on the muscle post-injury regeneration process from a functional, histological and molecular point of view. Our results demonstrate that animals submitted to IHH recovered the functionality of the injured muscle in only 9 days, while animals exposed to ICE and to hypoxia and cold simultaneously (COHY) needed 21 days to achieve the restoration of muscle function. The histological markers of muscle regeneration indicated that IHH improved the regeneration process, as evidenced by the reduction of dMHC, the presence of centrally nucleated fibres and collagen I deposition after 9 days of treatment, while this amelioration was not shown in the groups subjected to intermittent cold (COLD and COHY). The overexpression of pSer473Akt to total Akt in all treated groups at 9 days and the overexpression of pThr172AMPKα to total AMPKα in the HYPO group 21 days after injury are the main indicators of changes in the muscle healing process.

Functional recovery

Muscle injury is characterized by the loss of muscle function (Tidball, 2011; Tiidus, 2008; Warren et al., 1999), with deficiencies appearing rapidly in the first few hours after injury (within the first 24 h) (Ingalls et al., 1998). The functional impairment is maintained throughout the muscle regeneration process and is still detectable 10–21 days after the injury (Contreras-Muñoz et al., 2016, 2017, 2021; Head et al., 2014; Pereira et al., 2014). Injuries induce alterations in the muscle excitation–contraction coupling, which translate into reductions of around 37–50% of muscle tetanic force during the first 3 days after injury (Ingalls et al., 1998). In addition, it has been described that the greatest muscular deficiencies after injury are much more marked when the muscles are stimulated at low frequencies (around 20 Hz) than at high frequencies (above 50 Hz). Thereby, low-frequency fatigue, characterized by reduced contractile activation and peripheral failure, is used as an important parameter to assess muscle injury and its regeneration process (Tiidus, 2008). To ensure that our muscle injury protocol was efficient in inducing muscle damage, we assessed several functional and morphological properties 24 h following injury (CTRL_0 group). Our results demonstrate that the surgical procedure used induced a significant functional disturbance (Fig. 1) and clearly

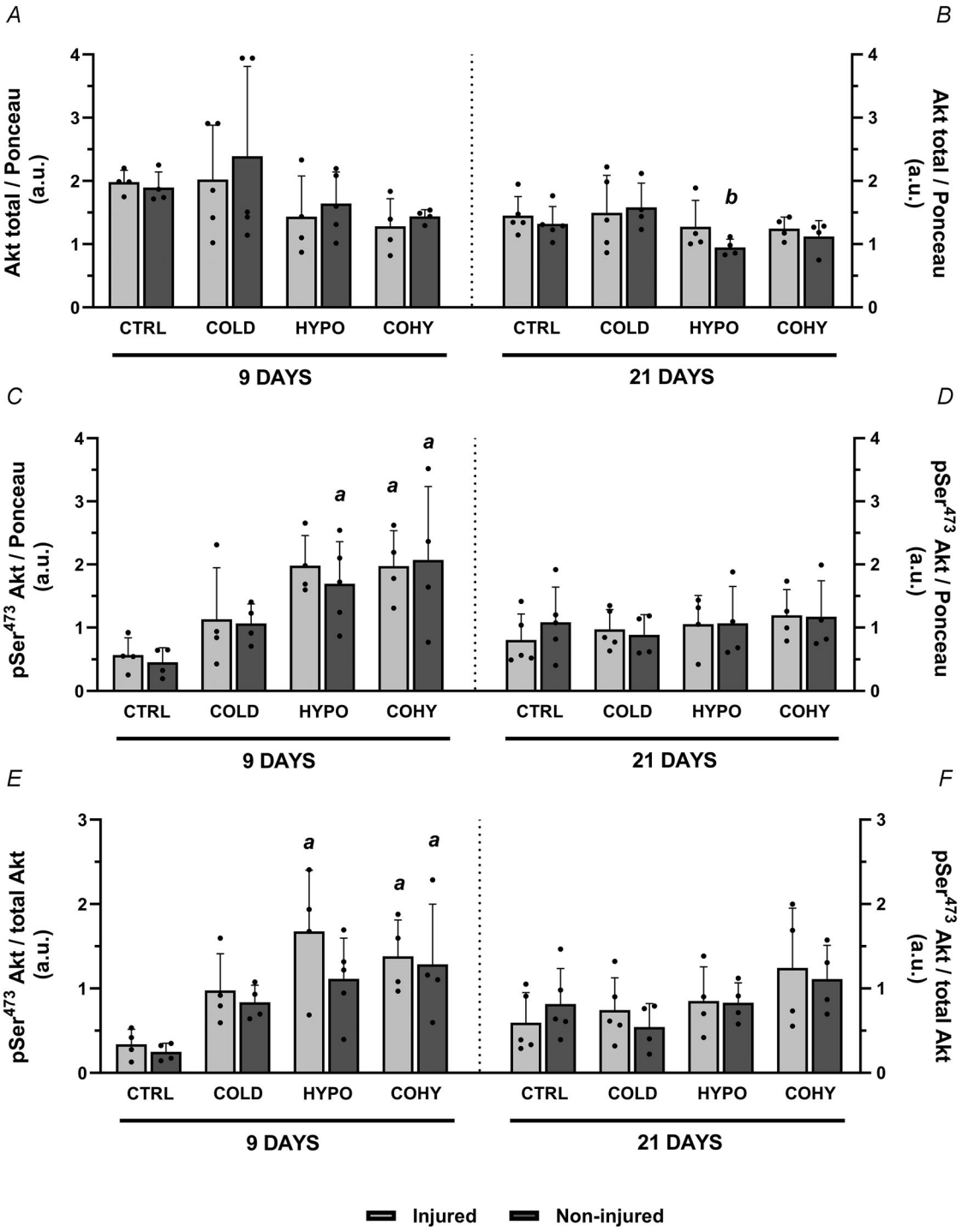


Figure 6. Expression of Akt and pSer473Akt proteins in the injured and non-injured gastrocnemius muscles

A and *B*, total Akt; *C* and *D*, pSer473Akt; and *E* and *F*, pSer473Akt to total Akt ratio. Data are presented as mean ± standard deviation and expressed in arbitrary units (a.u.). Points represent the values of each sample; n = 4–5 per group at each time point. Statistically significant differences are indicated as: *B*, [b]Non-Injured: HYPO *vs*. COLD: $P = 0.011$. *C*, [a]Injured: COHY *vs*. CTRL: $P = 0.027$; Non-Injured: HYPO *vs*. CTRL: $P = 0.046$ and COHY *vs*. CTRL: $P = 0.009$. *E*, [a]Injured: HYPO *vs*. CTRL: $P = 0.015$ and COHY *vs*. CTRL: $P = 0.024$; Non-Injured: COHY *vs*. CTRL: $P = 0.025$. CTRL, control; COLD, intermittent cold; HYPO, intermittent hypobaric hypoxia; COHY, simultaneous intermittent cold and hypobaric hypoxia.

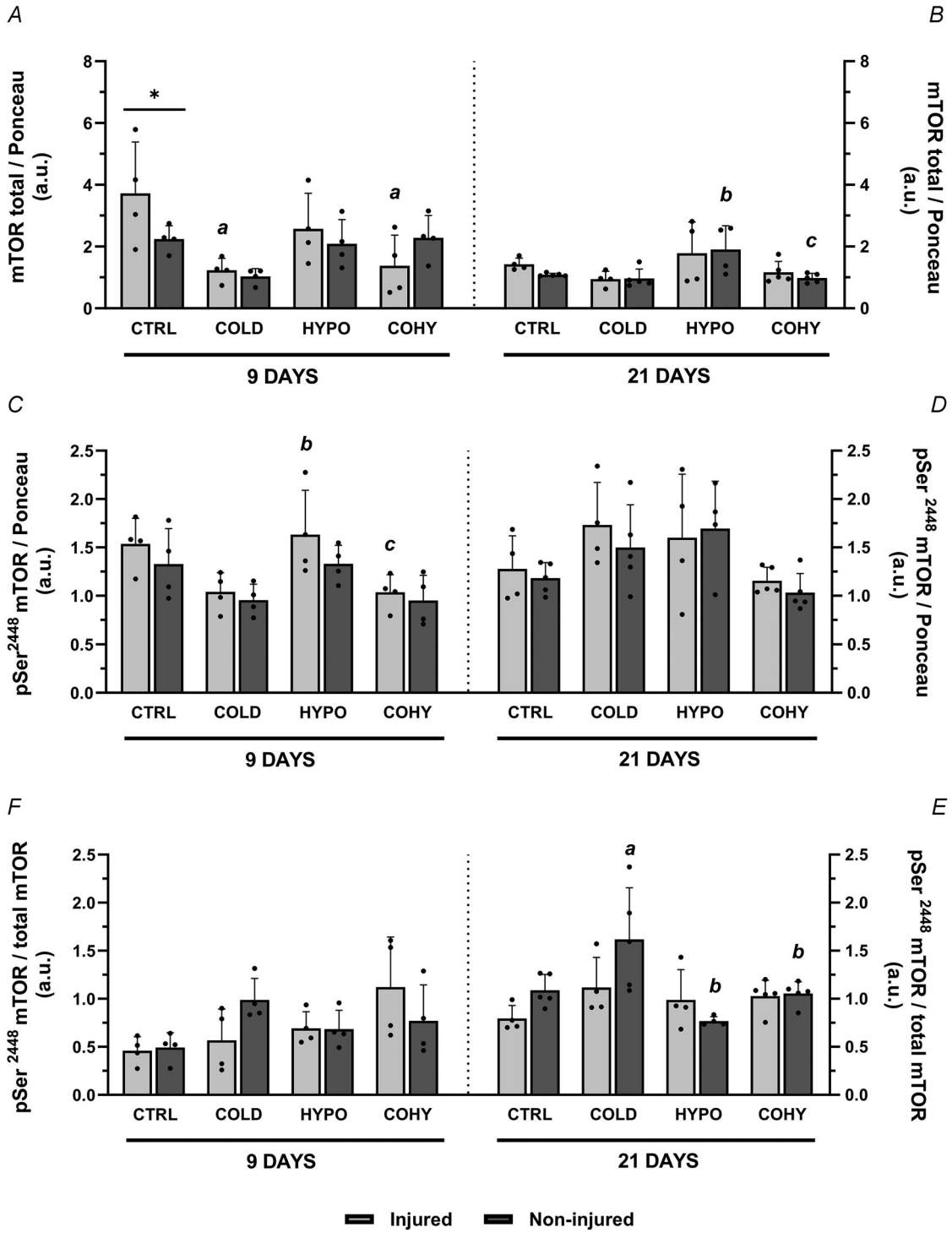


Figure 7. Expression of mTOR and pSer2448mTOR proteins in injured and non-injured gastrocnemius muscles

A and *B*, total mTOR; *C* and *D*, pSer2448mTOR; and *E* and *F*, pSer2448mTOR to total mTOR ratio. Data are presented as mean ± standard deviation and expressed in arbitrary units (a.u.). Points represent the values of each sample; $n = 4–5$ per group at each time point. Statistically significant differences are indicated as: *A*, [a]Non-Injured: COLD *vs*. CTRL: $P = 0.004$ and COHY *vs*. CTRL: $P = 0.007$. *B*, [b]Non-Injured: HYPO *vs*. COLD: $P = 0.035$; [c]COHY *vs*. HYPO: $P = 0.039$. *C*, [b]Injured: HYPO *vs*. COLD: $P = 0.041$; [c]COHY *vs*. COLD: $P = 0.039$. *E*, [a]Non-Injured: COLD *vs*. CTRL: $P = 0.030$; [b]HYPO *vs*. COLD: $P < 0.001$ and COHY *vs*. COLD: $P = 0.019$. CTRL, control; COLD, intermittent cold; HYPO, intermittent hypobaric hypoxia; COHY, simultaneous intermittent cold and hypobaric hypoxia.

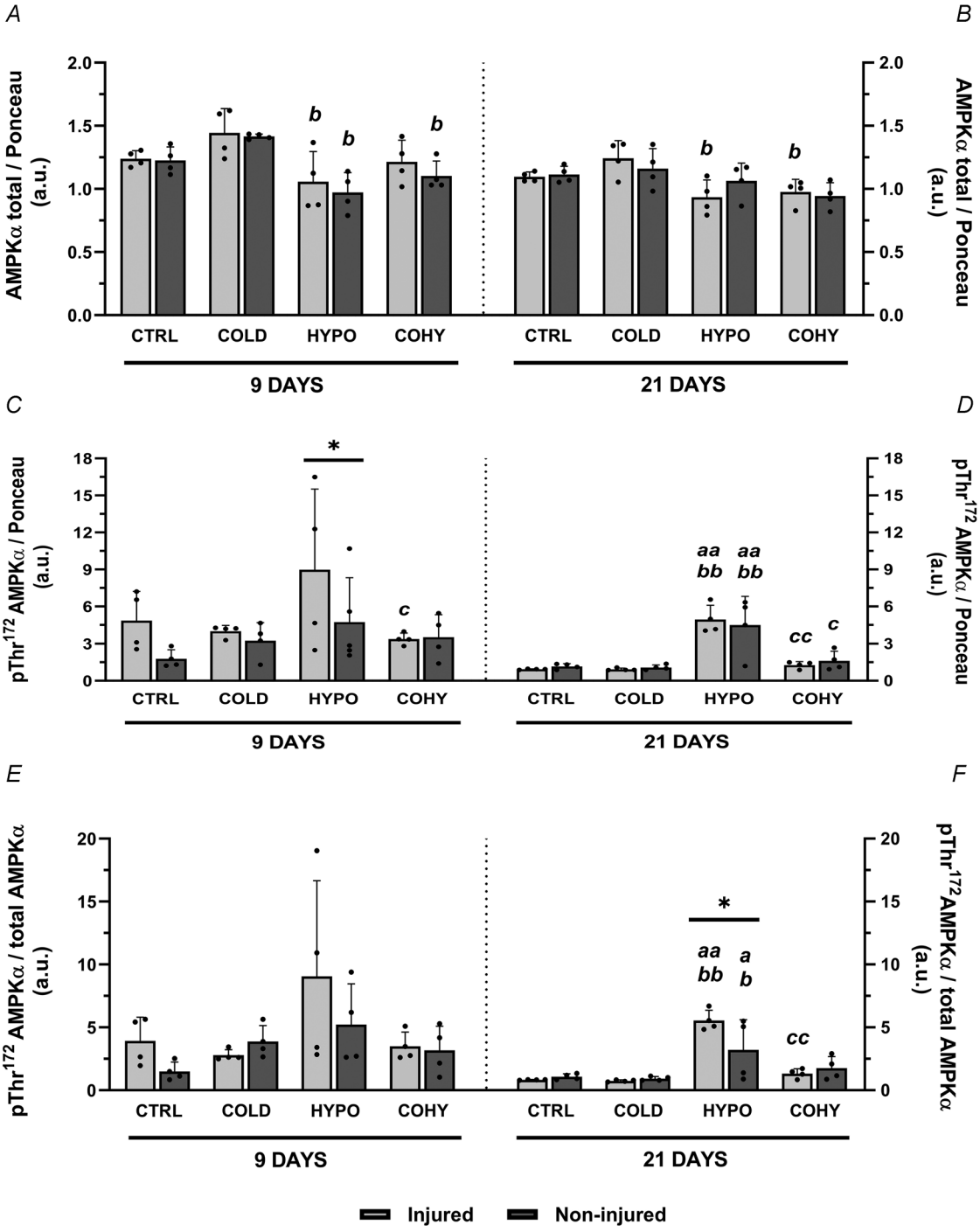


Figure 8. Expression of AMPKα and pThr172AMPKα proteins in injured and non-injured gastrocnemius muscles

A and *B*, total AMPKα; *C* and *D*, pThr172AMPKα; and *E* and *F*, pThr172AMPKα to total AMPKα ratio. Data are presented as mean ± standard deviation and expressed in arbitrary units (a.u.). Points represent the values of each sample; n = 4–5 per group at each time point. Statistically significant differences are indicated as: *A*, [b]HYPO *vs*. COLD $P = 0.007$. *B*, [b]Injured: HYPO *vs*. COLD: $P = 0.006$ and COHY *vs*. COLD: $P = 0.022$. *C*, *Injured *vs*. Non-Injured: HYPO: $P = 0.007$, [c]Non-Injured: COHY *vs*. HYPO: $P = 0.041$. *D*, [a]Injured: HYPO *vs*. CTRL: $P < 0.001$; Non-Injured: HYPO *vs*. CTRL: $P < 0.001$; [b]Injured: HYPO *vs*. COLD: $P < 0.001$; Non-Injured: HYPO *vs*. COLD: $P < 0.001$; [c]Injured: COHY *vs*. HYPO: $P < 0.001$; Non-Injured: COHY *vs*. HYPO: $P = 0.003$. *F*, [a]Injured: HYPO *vs*. CTRL: $P < 0.001$; Non-Injured: HYPO *vs*. CTRL: $P < 0.030$; [b]Injured: HYPO *vs*. COLD: $P < 0.001$; Non-Injured: HYPO *vs*. COLD: $P = 0.018$; [c]Injured: COHY *vs*. HYPO: $P < 0.001$. CTRL, control; COLD, intermittent COLD; HYPO, intermittent hypobaric hypoxia; COHY, simultaneous intermittent cold and hypobaric hypoxia.

identifiable structural damage at the microscopic level (Fig. 2). Moreover, our findings also demonstrate that the regeneration process from muscle damage in our animal model is not functionally complete after 21 days, since PF, TetF and LFF were significant lower in injured GAS (Fig. 3*B*, *D* and *F*).

Pharmacological and non-pharmacological therapies are oriented to accelerate muscle regeneration (Laumonier & Menetrey, 2016) and to recover muscle function as fast as possible. In the current investigation, we used intermittent exposure to cold and hypoxia as therapies to accelerate the muscle regeneration process. Our results showed that animals exposed to IHH (HYPO) presented a recovery of the muscular capacities in the injured GAS muscle (PF, TetF and LFF) after 9 days of treatment (Fig. 3*A*, *C* and *E*), matching its contralateral un-injured muscle. In contrast, animals exposed to intermittent cold (COLD) or exposed simultaneously to intermittent cold and hypoxia (COHY) needed 21 days of treatment to reach a functional recovery equivalent to their contralateral muscle (Fig. 3).

To the best of our knowledge, there is no previous work describing the effects of intermittent cold and hypoxia, simultaneously or separately, on muscle function recovery after injury. Nevertheless, some researchers have studied the effect of hypobaric hypoxia exposure on non-injured

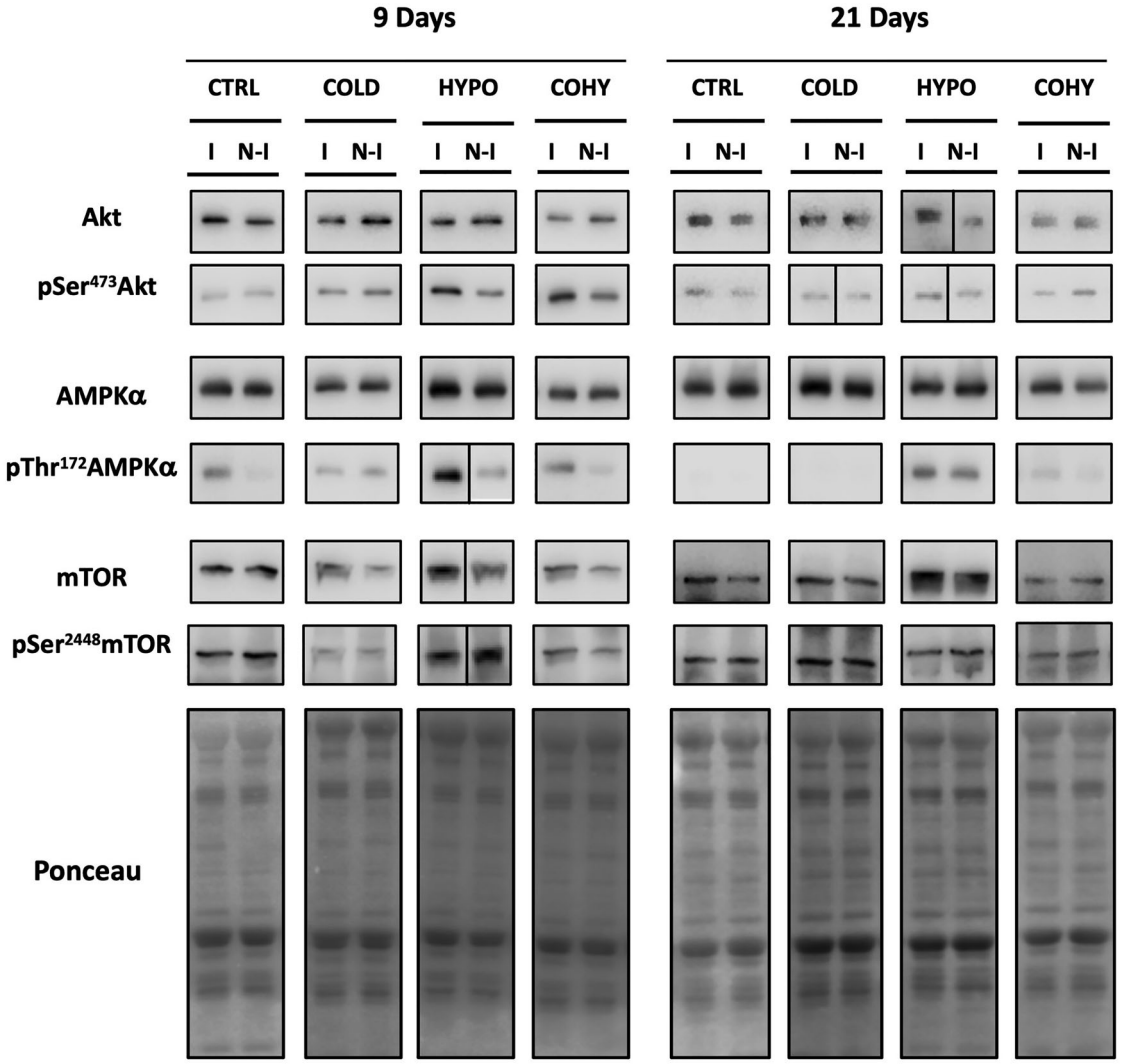


Figure 9. Representative images of the expression of Akt, pSer473Akt, AMPKα, pThr172AMPKα, mTOR and pSer2448mTOR proteins in injured (I) and non-injured (N-I) gastrocnemius muscles, and an example of loading control (Ponceau staining)
The figure is composed of the combination of different bands coming from different membranes, as indicated by lines, that were selected as representative according to the results of the statistical analysis of our data. CTRL, control; COLD, intermittent COLD; HYPO, intermittent hypobaric hypoxia; COHY, simultaneous intermittent cold and hypobaric hypoxia.

muscle functional capacities. Itoh et al. (1990) reported that after chronic hypobaric hypoxia exposure (10 weeks at 4000 m) the soleus (SOL) muscle of rats did not present changes in PF nor fatigue, increased tetanic frequency, and showed reduced CT and HRT. However, in the same study, the muscle extensor digitorum longus (EDL) showed after hypoxic exposure reduced PF and TetF, increased resistance to fatigue, and no changes in HRT and CT compared to muscles not submitted to hypoxia (Itoh et al., 1990). Nonetheless, it should be noted that Itoh et al. (1990) observed significant changes in fibre type proportion [decrease of fast glycolytic (FG) fibres and increase of fast oxidative glycolytic (FOG) fibres in EDL and reduction of slow oxidative (SO) and increase of FOG fibres in SOL] and reduction of FCSA in the EDL muscle. Therefore, most of the observed changes in muscle force capacities could be directly related to the shift of fibre type and the reduction of FCSA produced by hypoxic exposure (Itoh et al., 1990). Later, Shiota et al. (2004) studied the responses of Sprague-Dawley rats for 6 weeks at 5000 m of altitude. This investigation showed in *in vitro* measurements that muscles exposed to hypobaric hypoxia produced less force when simulated at high frequencies, but they did not report significant changes in parameters such as PF, HRT, CT or fatigue in either the SOL or the EDL. Likewise, Faucher et al. (2005) submitted rats to normobaric hypoxia (5000 m) for 1 month and showed, also in *in vitro* measurements, that SOL increased TetF, accompanied by an increase of the proportion of type I fibres, while EDL registered a reduction of the TetF without changes in the proportion of type I fibres. More recently, another study reported that 6 weeks of hypobaric hypoxia (4000 m) increased PF and decreased resistance to the fatigue of both SOL and EDL and an increase in TetF in the SOL (El-Khoury et al., 2012).

In the current investigation, no differences were observed between HYPO and CTRL in the contractile functional properties of the non-injured GAS (Fig. 3), even though the HYPO group showed a tendency to smaller fibres and a shift from slow to fast fibres (Fig. 4). Therefore, taking our results and the previous pieces of evidence together, it appears that, from a functional point of view, skeletal muscle responses to a hypoxic stimulus depend on the dose of hypoxia (altitude and duration) and, when intermittent, also on the frequency of hypoxia exposure. In addition to these key factors, the type of experiment or measure (*in vivo versus in vitro*) and the muscle studied (EDL, SOL, GAS) could lead to different outcomes (Faucher et al., 2005). Furthermore, most of the studies reported changes in the fibre type proportion and in the FCSA of the studied muscles, these changes in muscle morphology being a plausible explanation of the observed changes in muscle function, due to the direct influence of fibre type and FCSA in force production (Huard et al., 2002).

Regarding the different treatments, we conclude from our results that the most efficient protocol for functional muscle recovery is IHH prescribed alone (HYPO) since it is the only protocol that recovers the muscle contractile properties after only 9 days of treatment. The use of intermittent cold, alone or in combination with IHH, needs more time to induce total functional recovery. We can speculate that the local vasoconstriction elicited in peripheral tissues by cold could cause a blunting effect counteracting the well-known local vasodilator effect of hypoxia.

Histological events after injury

Progress in regenerative medicine requires constantly searching for new therapies that accelerate muscle healing, but always considering that the main objective of the regeneration process is to obtain a complete recovery of muscle functionality, together with recovery of the previous muscle tissue architecture and mechanical properties to avoid re-injuries (Forcina et al., 2020; Huard et al., 2002).

Once the post-injury acute inflammatory response is over, the muscle regeneration process takes place. During the first week after injury, new muscle fibres begin to appear at the injury site, expressing dMHC until they mature, at which time the dMHC is replaced by the adult MHC isoform (Chargé & Rudnicki, 2004; Ciciliot & Schiaffino, 2010; Forcina et al., 2020; Shibaguchi et al., 2019). Developmental myosin (neonatal + embryonic) is only expressed during embryonic life but can be temporarily re-expressed during the process of muscle regeneration (Schiaffino et al., 2015; Shibaguchi et al., 2019; Whalen et al., 1990). Newly grown fibres express dMHC from the first 2–3 days after injury and this expression is still detectable until 2–3 weeks after injury, although, during a normal regeneration process, the percentage of dMHC-positive fibres decreases over time (Ciciliot & Schiaffino, 2010; Contreras-Muñoz et al., 2016, 2017; Forcina et al., 2020). Our results in CTRL muscles agree with these findings: dMHC was present in almost 50% of the fibres on day 9 following injury but declined to 15% of the fibres on day 21 (Fig. 5*B*). At the initial stages of the regeneration process, the newly formed fibres had central nuclei and small FCSA and, as a result of the maturation process, the central nuclei of the newly formed fibres migrated to the periphery as the fibre increased in size, finally leading to mature fibres with peripheral nuclei and the size of fully developed muscle fibres (Chargé & Rudnicki, 2004; Ciciliot & Schiaffino, 2010; Forcina et al., 2020). These previously reported events are in agreement with those observed in our CTRL group (Fig. 5*C* and *D*). Consequently, regardless of whether muscles were treated or not, the skeletal muscle of the rats had almost repaired at the histological level by day 21 (Fig. 5), as previously

described (Ciciliot & Schiaffino, 2010; Contreras-Muñoz et al., 2016, 2017; Forcina et al., 2020).

Looking at the effect of our treatments, the results showed that 9 days after injury the fastest regeneration was achieved by HYPO, as indicated by the reduced proportion of dMHC-positive fibres and the lower proportion of fibres with central nuclei (Fig. 5*B* and *D*). However, the HYPO group exhibited newly formed fibres with smaller FCSA on day 9, suggesting that the few dMHC-positive fibres observed at this time point had a delayed growth (Fig. 5*C*). Thus, 9 days after injury the HYPO group presented similar histological outcomes (percentage dMHC-positive fibres, FCSA, central nuclei and collagen I deposition) to those observed after 21 days in CTRL (Fig. 5*B*–*D*). Nevertheless, these results contrast with the findings reported by Chaillou, Koulmann, Meunier, Pugnière, et al. (2014) who did not observe changes in the percentage of fibres expressing immature isoforms of myosin (neonatal and embryonic) 7 days after injury in SOL subjected to chronic hypobaric hypoxia (5500 m). Again, these discrepancies in the results could be related to the dose (4500 *vs*. 5500 m) and type of hypoxia (intermittent *vs*. chronic). Thus, IHH improved the muscle regeneration process, while this was not observed with chronic hypoxia (Chaillou, Koulmann, Meunier, Pugnière, et al., 2014). IHH, in contrast to chronic hypoxia, preserves muscle mass (see Table 1) and improves muscle irrigation, and hence oxygen delivery to muscles (Santocildes et al., 2021). We can speculate that an enhanced oxygen supply to the muscle tissue derived from intermittent hypoxic exposure could explain the fastest regeneration of the injured GAS.

The local application of cold has been widely used as a therapeutic tool for muscle injury recovery (Malanga et al., 2015; Nadler et al., 2004; Shibaguchi et al., 2016, 2019; Vieira Ramos et al., 2016). However, previous studies did not demonstrate changes in the proliferation and differentiation of satellite cells, nor in the expression of desmin after cold treatments (Shibaguchi et al., 2016; Vieira Ramos et al., 2016). Moreover, and in contrast to our findings (Fig. 5*B* and *D*), these previous works did not report changes in neonatal and embryonic myosin expression in the injured area nor the percentage of centrally nucleated fibres (Shibaguchi et al., 2019; Vieira Ramos et al., 2016). Thus, it seems that the application of local cold did not modify the muscle regeneration process at the histological level (Shibaguchi et al., 2016, 2019; Vieira Ramos et al., 2016). Contrasting with these previous findings, here we applied a protocol of intermittent cold, alone (COLD) or in combination with IHH (COHY), to the whole animal, and found that although the changes in the percentage of fibres expressing dMHC and central nuclei were not as pronounced as in HYPO, the histological muscle regeneration after 9 days following injury was accelerated when compared to CTRL (Fig. 5*B* and *D*). As occurred with the IHH, the previously identified up-regulation of vascular endothelial growth factor (VEGF) and consequent increased capillarization of the muscle tissue after ICE treatments in rats could explain this outcome (Santocildes et al., 2021).

The muscle repair process begins with the deposition of extracellular matrix (ECM) at the injury site. ECM deposition begins during the first few days after injury and is mediated by fibroblast activity in response to various growth factors such as TGF-β1 (Forcina et al., 2020; Laumonier & Menetrey, 2016; Li et al., 2001; Souza & Gottfried, 2013; Turner & Badylak, 2012). Fibronectin and collagen III are among the first proteins expressed by the ECM during the repair process, whereas collagen I is activated later and remains active during the subsequent weeks (Kääriäinen et al., 2000). Although collagen deposition has a physiological function in helping to maintain muscle architecture after injury, its excessive production can lead to fibrosis, hindering optimal tissue repair and muscle functionality and increasing the risk of re-injury (Forcina et al., 2020; Laumonier & Menetrey, 2016; Souza & Gottfried, 2013). Thus, together with the activation of satellite cells, the control of collagen deposition is essential in the regeneration process, since the excess of connective tissue could compromise the availability of growth factors and the migration of satellite cells (Vieira Ramos et al., 2016). Thus, reducing muscle fibrosis is one of the main objectives of the different treatments applied to improve muscle regeneration. Anti-fibrotic agents, platelet-rich plasma, early mobilization and light exercise are some examples of procedures used to prevent excessive collagen deposition at the injury site (Contreras-Muñoz et al., 2017; Huard et al., 2002). To the best of our knowledge, this is the first time that the effects of hypoxia on post-injury collagen deposition have been studied. Our results indicate that the use of IHH (HYPO and COHY) reduced collagen I deposition at the injury site, in both the early and the late phases of regeneration (Fig. 5*E*). Regarding the effect of cold therapy, previous studies have shown that a single application (20 min) of local cold after injury produced both short-term (7 days) and medium-term (15 days) increases in collagen deposition, but without producing alterations in the infiltration of macrophages, proliferation and differentiation of satellite cells, or in the expression of immature myosin (Shibaguchi et al., 2016, 2019). Vieira Ramos et al. (2016) reported that intermittent application of local cold (30 min, three times/day) reduced the inflammatory process, but without observing improvements in the repair process, nor in the deposition of collagen I/III. In agreement with previous findings (Shibaguchi et al., 2016, 2019; Vieira Ramos et al., 2016), our animals submitted to intermittent cold (COLD) did not show any improvement in collagen I deposition at the injury site, either in the short or long term of the repair

time course (Fig. 5*E*). However, the results obtained after using IHH and cold simultaneously are confusing. Since collagen I deposition was reduced in COHY after the first 9 days of treatment, mimicking that observed in HYPO, an increase in collagen I deposition was observed after 21 days in COHY (Fig. 5*E*). The timing of TGF-β1 activity has been suggested as a key factor responsible for the accumulation or lack of improvement in the management of collagen deposition (Shibaguchi et al., 2016; Vieira Ramos et al., 2016). The results found in the HYPO group open an interesting field for research in the study of the modulation of TGF-β1 by intermittent hypoxic stimulus, as a lower collagen I deposition could have substantial benefits for skeletal muscle repair through reduced fibrosis.

Molecular responses

The Akt/mTOR signalling pathway is one of the most important pathways up-regulating muscle growth and hypertrophy through anabolic processes such as protein synthesis (Liu et al., 2018; Wei et al., 2019; Zhang et al., 2015). mTOR has an important role in the different steps of the muscle regeneration process: first, during the activation, proliferation and differentiation of MuSC, later in myoblast fusion, and finally in the growth of newly formed muscle fibres (Wei et al., 2019; Zhang et al., 2015). It has been reported (Chaillou, Koulmann, Meunier, Pugnière, et al., 2014; Liu et al., 2018; Pereira et al., 2014), and our results corroborate (Figs 6 and 7), that Akt and mTOR showed higher expression within the first week following injury and that these differences disappeared after 14 days. Akt is essential for the development, growth and regeneration of skeletal muscle and for maintaining its correct metabolism, but it also plays a key role in the differentiation and maintenance of the myoblast and myofibre maturation (Gardner et al., 2012). Thus, the improvement in muscle regeneration observed in COLD, HYPO and COHY after 21 days of treatment at functional (Fig. 3) and histological (Fig. 5) levels could have been mediated by Akt phosphorylation, which was overexpressed during the first 9 days after injury (Fig. 6*C* and *E*), initiating the molecular cascade of events leading to the functional and morphological improvements.

AMPK is the central sensor of the intracellular energy state. This kinase regulates the anabolic and catabolic pathways to maintain the balance between energy supply and demand (Kjøbsted et al., 2018). Acute AMPK activation enhances glucose transport and fatty acid oxidation while reducing glucose synthase and protein synthesis, through mTOR inhibition. The preference for a high-carbohydrate diet is common among climbers. The use of glucose as the main metabolic substrate during hypoxia is beneficial because by shifting the respiratory quotient from 0.7 to near 1, the alveolar partial pressure of oxygen is increased for a given CO_2 alveolar content, providing a remarkable gain in arterial saturation. Hypoxia stimulates the glycolytic flux and increases the availability of pyruvate, due in part to the elevated levels of adrenaline (Viscor et al., 2023; West et al., 2012). During cold exposure, skeletal muscle increases the oxygen and glucose uptake to maintain the high oxygen consumption and metabolism required to support thermogenesis. Glucose is mainly used through oxidative metabolism, which is upregulated by insulin-independent pathways (Sepa-Kishi et al., 2017). During the muscle regeneration process, AMPKα1 is involved in the skewing from a pro- to anti-inflammatory phenotype of the macrophages and in phagocytosis of the necrotic tissue by macrophages (McArthur et al., 2020; Mounier et al., 2013). Moreover, the absence of AMPKα1 in the MuSC is associated with reduced proliferation and differentiation of MuSC and increased fibrogenesis (Fu et al., 2015). In the current study, we did not observe changes in the expression of pThr172AMPK and AMPK proteins after muscle injury in the CTRL animals (Fig. 8*A*, *C* and *E*). Thus, our results agree with those reported by Chaillou, Koulmann, Meunier, Pugnière, et al. (2014) who reported a higher pThr172AMPK to total AMPK ratio 3 days after injury but did not show significant changes on days 7, 14 and 28 (Chaillou, Koulmann, Meunier, Pugnière, et al., 2014). Our results show that the phosphorylated form of AMPK (and its ratio to total AMPK) had highly significant differences in HYPO injured muscles compared to CTRL and the other treated groups after 21 days (Fig. 8). However, Chaillou, Koulmann, Meunier, Pugnière, et al. (2014) observed a significant increase in phosphorylated AMPK and its ratio to total AMPK as soon as 3 days after injury in animals treated with hypoxia, but they did not find greater values later throughout the muscle regeneration process. The discrepancies in the time course expression of this protein in animals treated with hypoxia could be due to the different protocols of hypoxia used (dose and time of exposure). Regardless, we can speculate that the increase of pThr172AMPK and pThr172AMPK to total AMPK ratio observed after hypoxic treatment (HYPO) could be involved in the inflammatory response and the shift of macrophage phenotype during the first steps of the muscle regeneration process (McArthur et al., 2020; Mounier et al., 2013), helping to aid MuSC proliferation and differentiation and thus reducing fibrosis at the injury site (Fu et al., 2015).

Regarding the effects of hypoxia in the regulation of muscle mass and muscle metabolism, previous studies have shown that chronic hypobaric hypoxia (5500 m) did not produce changes in pThr172AMPK to total AMPK nor after short (3–7 days) or long-term (14–28 days) exposure (Chaillou et al., 2012; Chaillou, Koulmann, Meunier, Chapot, et al., 2014). However, although we have not observed changes in pThr172AMPK to total

AMPK ratio after 9 days of IHH, higher expression has been shown after 21 days of exposure (Fig. 8*E* and *F*). In agreement with what we observed here, some previous reports did not find changes in pSer473Akt to total Akt ratio both after IHH and chronic hypoxia, neither after short-term (5–12 days) nor after long-term (30 days) exposure (Chaillou et al., 2012; Chaillou, Koulmann, Meunier, Pugnière, et al., 2014; Siques et al., 2018). On the other hand, no changes were observed in pSer2448mTOR to total mTOR ratio during intermittent exposure to hypoxia, and thus intermittent exposure to hypoxia seems to avoid the negative effects of chronic hypoxia on muscle mass regulation and in the muscle regeneration process (Fig. 7*E*) (Chaillou et al., 2012).

In conclusion, contrary to our hypothesis, the combination of cold and hypoxia has blunted the beneficial effects elicited by IHH to improve muscle recovery. Furthermore, cold was not able to achieve the results obtained by IHH. All treated animals recovered muscle function and regenerated muscle histological structure 21 days following injury, contrasting with the non-treated animals which still showed signs of muscle functional and histological alterations at this time. The increased expression of the phosphorylated form of Akt found after 9 days in all treated groups could have initiated the molecular cascade of events leading to these functional and morphological improvements after 21 days. Among all treatments, intermittent hypobaric exposure was the only one that accelerated muscle recovery as early as 9 days after injury. The overexpression of phosphorylated AMPKα initiated after 9 days and hugely increased in intermittent hypoxia-treated animals after 21 days could be involved in the initial inflammatory response, thus accelerating the muscle regeneration process.

This study provides solid evidence of the role that intermittent exposure to hypobaric hypoxia has in the regeneration of skeletal muscle tissue after injury. Therefore, it represents an important contribution to new ways of applying hypoxia exposure programmes that have interesting applications in the biomedical field. Exposure to hypoxia could prove useful not only in training schedules for elite and amateur athletes, as it has been used until now, but also in some pathophysiological conditions such as recovery from musculoskeletal injuries or even delaying sarcopenia or recovering from long COVID syndrome. Thus, a wide field of research of great interest is opening up in the sports, health and technological sectors.

REFERENCES

Allen, D. G., Lamb, G. D., & Westerblad, H. (2008). Skeletal muscle fatigue: Cellular mechanisms. *Physiological Reviews*, **88**(1), 287–332.

American Veterinary Medical Association. (2020). *AVMA guidelines for the euthanasia of animals: 2020 edition*. Schaumburg. United States of America.

Beidleman, B. A., Fulco, C. S., Staab, J. E., Andrew, S. P., & Muza, S. R. (2014). Cycling performance decrement is greater in hypobaric versus normobaric hypoxia. *Extreme Physiology & Medicine*, **3**, 8.

Boos, C. J., O'Hara, J. P., Mellor, A., Hodkinson, P. D., Tsakirides, C., Reeve, N., Gallagher, L., Green, N. D. C., & Woods, D. R. (2016). A four-way comparison of cardiac function with normobaric normoxia, normobaric hypoxia, hypobaric hypoxia and genuine high altitude. *PLoS ONE*, **11**(4), 1–17.

Bourdillon, N., Aebi, M. R., Kayser, B., Bron, D., & Millet, G. P. (2023). Both hypoxia and hypobaria impair baroreflex sensitivity but through different mechanisms. *International Journal of Sports Medicine*, **44**(3), 177–183.

Burtscher, J., Mallet, R. T., Pialoux, V., Millet, G. P., & Burtscher, M. (2022). Adaptive responses to hypoxia and/or hyperoxia in humans. *Antioxidants & Redox Signaling*, **37**(13–15), 887–912.

Chaillou, T., Koulmann, N., Meunier, A., Chapot, R., Serrurier, B., Beaudry, M., & Bigard, X. (2014). Effect of hypoxia exposure on the recovery of skeletal muscle phenotype during regeneration. *Molecular and Cellular Biochemistry*, **390**(1–2), 31–40.

Chaillou, T., Koulmann, N., Meunier, A., Pugnière, P., McCarthy, J. J., Beaudry, M., & Bigard, X. (2014). Ambient hypoxia enhances the loss of muscle mass after extensive injury. *Pflugers Archiv: European journal of physiology*, **466**(3), 587–598.

Chaillou, T., Koulmann, N., Simler, N., Meunier, A., Serrurier, B., Chapot, R., Peinnequin, A., Beaudry, M., & Bigard, X. (2012). Hypoxia transiently affects skeletal muscle hypertrophy in a functional overload model. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, **302**(5), R643–R654.

Chargé, S. B. P., & Rudnicki, M. A. (2004). Cellular and molecular regulation of muscle regeneration. *Physiological Reviews*, **84**(1), 209–238.

Ciciliot, S., & Schiaffino, S. (2010). Regeneration of mammalian skeletal muscle: Basic mechanisms and clinical implications. *Current Pharmaceutical Design*, **16**(8), 906–914.

Contreras-Muñoz, P., Fernández-Martín, A., Torrella, J. R., Serres, X., De, L.a Varga, M., Viscor, G., Järvinen, T. A. H., Martínez-Ibáñez, V., Peiró, J. L., Rodas, G., & Marotta, M. (2016). A new surgical model of skeletal muscle injuries in rats reproduces human sports lesions. *International Journal of Sports Medicine*, **37**(3), 183–190.

Contreras-Muñoz, P., Torrella, J. R., Serres, X., Rizo-Roca, D., De La Varga, M., Viscor, G., Martínez-Ibáñez, V., Peiró, J. L., Järvinen, T. A. H., Rodas, G., & Marotta, M. (2017). Postinjury exercise and platelet-rich plasma therapies improve skeletal muscle healing in rats but are not synergistic when combined. *American Journal of Sports Medicine*, **45**(9), 2131–2141.

Contreras-Muñoz, P., Torrella, J. R., Venegas, V., Serres, X., Vidal, L., Vila, I., Lahtinen, I., Viscor, G., Martínez-Ibáñez, V., Peiró, J. L., Järvinen, T. A. H., Rodas, G., & Marotta, M. (2021). Muscle precursor cells enhance functional muscle recovery and show synergistic effects with post-injury treadmill exercise in a muscle injury model in rats. *American Journal of Sports Medicine*, **49**(4), 1073–1085.

Coppel, J., Hennis, P., Gilbert-Kawai, E., & Grocott, M. P. W. (2015). The physiological effects of hypobaric hypoxia versus normobaric hypoxia: A systematic review of cross-over trials. *Extreme Physiology & Medicine*, **4**, 1–20.

Dipasquale, D. M. (2017). Moving the debate forward: Are normobaric and hypobaric hypoxia interchangeable in the study of altitude? *Current Sports Medicine Reports*, **16**(2), 68–70.

Dueweke, J. J., Awan, T. M., & Mendias, C. L. (2017). Regeneration of skeletal muscle after eccentric injury. *Journal of Sport Rehabilitation*, **26**(2), 171–179.

Edouard, P., Branco, P., & Alonso, J. M. (2016). Muscle injury is the principal injury type and hamstring muscle injury is the first injury diagnosis during top-level international athletics championships between 2007 and 2015. *British Journal of Sports Medicine*, **50**(10), 619–630.

El-Khoury, R., Bradford, A., & O'halloran, K. D. (2012). Chronic hypobaric hypoxia increases isolated rat fast-twitch and slow-twitch limb muscle force and fatigue. *Physiological Research*, **61**(2), 195–201.

Faucher, M., Guillot, C., Marqueste, T., Kipson, N., Mayet-Sornay, M. H., Desplanches, D., Jammes, Y., & Badier, M. (2005). Matched adaptations of electro-physiological, physiological, and histological properties of skeletal muscles in response to chronic hypoxia. *Pflugers Archiv: European journal of physiology*, **450**(1), 45–52.

Forcina, L., Cosentino, M., & Musarò, A. (2020). Mechanisms regulating muscle regeneration: Insights into the inter-related and time-dependent phases of tissue healing. *Cells*, **9**(5), 1297.

Fu, X., Wang, H., & Hu, P. (2015). Stem cell activation in skeletal muscle regeneration. *Cellular and Molecular Life Sciences*, **72**(9), 1663–1677.

Fulco, C. S., Beidleman, B. A., & Muza, S. R. (2013). Effectiveness of preacclimatization strategies for high-altitude exposure. *Exercise and Sport Sciences Reviews*, **41**(1), 55–63.

Gardner, S., Anguiano, M., & Rotwein, P. (2012). Defining Akt actions in muscle differentiation. *American Journal of Physiology-Cell Physiology*, **303**(12), C1292–C1300.

Girard, O., Koehle, M., Maclniss, M., Guenette, J., Koelhe, M., Verges, S., Rupp, T., Jubeau, M., Perrey, S., Millet, G., Chapman, R., Levine, B., Conkin, J., Wessel, J., Nespoulet, H., Wuyam, B., Tamisier, R., Verges, S., Levy, P., … Nelatury, C. (2012). Comments on Point:Counterpoint: Hypobaric hypoxia does not induce different responses from normobaric hypoxia. *Journal of Applied Physiology*, **112**(10), 1788–1974.

Hamilton, B., Valle, X., Rodas, G., Til, L., Pruna-Grive, R., Gutierrez-Rincon, J. A., & Tol, J. L. (2015). Classification and grading of muscle injuries: A narrative review. *British Journal of Sports Medicine*, **49**(5), 306.

Hauser, A., Schmitt, L., Troesch, S., Saugy, J. J., Cejuela-Anta, R., Faiss, R., Robinson, N., Wehrlin, J. P., & Millet, G. P. (2016). Similar hemoglobin mass response in hypobaric and normobaric hypoxia in athletes. *Medicine and Science in Sports and Exercise*, **48**(4), 734–741.

Head, S. I., Houweling, P. J., Chan, S., Chen, G., & Hardeman, E. C. (2014). Properties of regenerated mouse extensor digitorum longus muscle following notexin injury. *Experimental Physiology*, **99**(4), 664–674.

Huard, J., Li, Y., & Fu, F. H. (2002). Muscle injuries and repair: Current trends in research. *Journal of Bone and Joint Surgery. American Volume*, **84**(5), 822–832.

Ingalls, C. P., Warren, G. L., & Amstrong, R. (1998). Dissociation of force production from MHC and actin contents in muscles injured by eccentric contractions. *Journal of Muscle Research and Cell Motility*, **19**(3), 215–224.

Itoh, K., Moritani, T. I., Ishida, K., Hirofuji, C., Taguchi, S., & Itoh, M. (1990). Hypoxia-induced fibre type transformation in rat hindlimb muscles Histochemical and electro-mechanical changes. *European Journal of Applied Physiology and Occupational Physiology*, **60**(5), 331–336.

Järvinen, T. A. H., Järvinen, M., & Kalimo, H. (2014). Regeneration of injured skeletal muscle after the injury. *Muscles, Ligaments and Tendons Journal*, **3**(4), 337–345.

Järvinen, T. A. H., Järvinen, T. L. N., Kääriäinen, M., Äärimaa, V., Vaittinen, S., Kalimo, H., & Järvinen, M. (2007). Muscle injuries: Optimising recovery. *Best Practice & Research: Clinical Rheumatology*, **21**(2), 317–331.

Kääriäinen, M., Järvinen, T., Järvinen, M., Rantanen, J., & Kalimo, H. (2000). Relation between myofibers and connective tissue during muscle injury repair. *Scandinavian Journal of Medicine & Science in Sports*, **10**(6), 332–337.

Kjøbsted, R., Hingst, J. R., Fentz, J., Foretz, M., Sanz, M. N., Pehmøller, C., Shum, M., Marette, A., Mounier, R., Treebak, J. T., Wojtaszewski, J. F. P., Viollet, B., & Lantier L. (2018). AMPK in skeletal muscle function and metabolism. *Federation of American Societies for Experimental Biology Journal*, **32**(4), 1741–1777.

Laumonier, T., & Menetrey, J. (2016). Muscle injuries and strategies for improving their repair. *Journal of Experimental Orthopaedics*, **3**(1), 15.

Li, Y., Cummins, J., & Huard, J. (2001). Muscle injury and repair. *Current Opinion in Orthopedics*, **12**(5), 409–415.

Liu, X., Zeng, Z., Zhao, L., Xiao, W., & Chen, P. (2018). Changes in inflammatory and oxidative stress factors and the protein synthesis pathway in injured skeletal muscle after contusion. *Experimental and Therapeutic Medicine*, **15**(2), 2196–2202.

Luks, A. M., Ainslie, P. N., Lawley, J. S., Roach, R. C., & Simonson, T. S. (2021). *High Altitude Medicine and Physiology* (6th edn). CRC Press.

Lundby, C., Calbet, J. A. L., & Robach, P. (2009). The response of human skeletal muscle tissue to hypoxia. *Cellular and Molecular Life Sciences*, **66**(22), 3615–3623.

Maffulli, N., Oliva, F., Frizziero, A., Nanni, G., Barazzuol, M., Via, A. G., Ramponi, C., Brancaccio, P., Lisitano, G., Rizzo, D., Freschi, M., Galletti, S., Melegati, G., Pasta, G., Testa, V., Valent, A., & Del Buono A. (2014). ISMuLT guidelines for muscle injuries. *Muscles, Ligaments and Tendons Journal*, **3**(4), 241–249.

Malanga, G. A., Yan, N., & Stark, J. (2015). Mechanisms and efficacy of heat and cold therapies for musculoskeletal injury. *Postgraduate Medicine*, **127**(1), 57–65.

Malliaropoulos, N., Isinkaye, T., Tsitas, K., & Maffulli, N. (2011). Reinjury after acute posterior thigh muscle injuries in elite track and field athletes. *American Journal of Sports Medicine*, **39**(2), 304–310.

McArthur, S., Juban, G., Gobbetti, T., Desgeorges, T., Theret, M., Gondin, J., Toller-Kawahisa, J. E., Reutelingsperger, C. P., Chazaud, B., Perretti, M., & Mounier, R. (2020). Annexin A1 drives macrophage skewing to accelerate muscle regeneration through AMPK activation. *Journal of Clinical Investigation*, **130**(3), 1156–1167.

Millet, G. P., Faiss, R., & Pialoux, V. (2012). Point: Hypobaric hypoxia induces/does not induce different responses from normobaric hypoxia. *Journal of Applied Physiology*, **112**(10), 1783–1784.

Millet, G. P., Faiss, R., & Pialoux, V. (2013). Evidence for differences between hypobaric and normobaric hypoxia is conclusive. *Exercise and Sport Sciences Reviews*, **41**(2), 133.

Mittal, M., Siddiqui, M. R., Tran, K., Reddy, S. P., & Malik, A. B. (2014). Reactive oxygen species in inflammation and tissue injury. *Antioxidants & Redox Signaling*, **20**(7), 1126–1167.

Mounier, R., & Brugniaux, J. V. (2012). Counterpoint: Hypobaric hypoxia does not induce different responses from normobaric hypoxia. *Journal of Applied Physiology*, **112**(10), 1784–1786.

Mounier, R., Théret, M., Arnold, L., Cuvellier, S., Bultot, L., Göransson, O., Sanz, N., Ferry, A., Sakamoto, K., Foretz, M., Viollet, B., & Chazaud, B. (2013). AMPKα1 regulates macrophage skewing at the time of resolution of inflammation during skeletal muscle regeneration. *Cell Metabolism*, **18**(2), 251–264.

Mueller-Wohlfahrt, H. W., Haensel, L., Mithoefer, K., Ekstrand, J., English, B., McNally, S., Orchard, J., Van Dijk, N., Kerkhoffs, G. M., Schamasch, P., Blottner, D., Swaerd, L., Goedhart, E., & Ueblacker, P. (2013). Terminology and classification of muscle injuries in sport: The Munich consensus statement. *British Journal of Sports Medicine*, **47**(6), 342–350.

Nadler, S. F., Weingand, K., & Kruse, R. J. (2004). The physiologic basis and clinical applications of cryotherapy and thermotherapy for the pain practitioner. *Pain Physician*, **7**(3), 395–399.

Núñez-Espinosa, C., Ferreira, I., Ríos-Kristjánsson, J. G., Rizo-Roca, D., García Godoy, M. D., Rico, L. G., Rubi-Sans, G., Torrella, J. R., Pagès, T., Petriz, J., & Viscor, G. (2015). Effects of intermittent hypoxia and light aerobic exercise on circulating stem cells and side population, after strenuous eccentric exercise in trained rats. *Current Stem Cell Research & Therapy*, **10**(2), 132–139.

Pereira, M. G., Baptista, I. L., Carlassara, E. O. C., Moriscot, A. S., Aoki, M. S., & Miyabara, E. H. (2014). Leucine supplementation improves skeletal muscle regeneration after cryolesion in rats. *PLoS ONE*, **9**(1), e85283–e85283.

Ramos-Romero, S., Santocildes, G., Piñol-Piñol, D., Rosés, C., Pagés, T., Hereu, M., Amézqueta, S., Torrella, J. R., Torres, J. L., & Viscor, G. (2020). Implication of gut microbiota in the physiology of rats intermittently exposed to cold and hypobaric hypoxia. *PLoS ONE*, **15**(11), e0240686–e0240686.

Rizo-Roca, D., Ríos-Kristjánsson, J. G., Núñez-Espinosa, C., Santos-Alves, E., Gonçalves, I. O., Magalhães, J., Ascensão, A., Pagès, T., Viscor, G., & Torrella, J. R. (2017). Intermittent hypobaric hypoxia combined with aerobic exercise improves muscle morphofunctional recovery after eccentric exercise to exhaustion in trained rats. *Journal of Applied Physiology*, **122**(3), 580–592.

Rizo-Roca, D., Ríos-Kristjánsson, J. G., Núñez-Espinosa, C., Santos-Alves, E., Magalhães, J., Ascensão, A., Pagès, T., Viscor, G., & Torrella, J. R. (2017). Modulation of mitochondrial biomarkers by intermittent hypobaric hypoxia and aerobic exercise after eccentric exercise in trained rats. *Applied Physiology, Nutrition and Metabolism*, **42**(7), 683–693.

Roman, W., Pinheiro, H., Pimentel, M. R., Segalés, J., Oliveira, L. M., García-Domínguez, E., Carmen Gómez-Cabrera, M., Serrano, A. L., Gomes, E. R., & Muñoz-Cánoves, P. (2021). Muscle repair after physiological damage relies on nuclear migration for cellular reconstruction. *Science (1979)*, **374**(6565), 355–359.

Santocildes, G., Merino, M., Fabiani, F., Pagès, T., Marotta, M., Viscor, G., & Torrella, J. R. (2022). Histomorphological and functional contralateral symmetry in the gastrocnemius muscles of the laboratory rat. *Journal of Anatomy*, **241**(3), 692–701.

Santocildes, G., Viscor, G., Pagès, T., Ramos-Romero, S., Torres, J. L., & Torrella, J. R. (2021). Physiological effects of intermittent passive exposure to hypobaric hypoxia and cold in rats. *Frontiers in Physiology*, **12**, 673095.

Savourey, G., Launay, J. C., Besnard, Y., Guinet, A., & Travers, S. (2003). Normo- and hypobaric hypoxia: Are there any physiological differences? *European Journal of Applied Physiology*, **89**(2), 122–126.

Schiaffino, S., Rossi, A. C., Smerdu, V., Leinwand, L. A., & Reggiani, C. (2015). Developmental myosins: Expression patterns and functional significance. *Skeleton Muscle*, **5**, 22.

Semenza, G. L. (1998). Hypoxia-inducible factor 1 and the molecular physiology of oxygen homeostasis. *Journal of Laboratory and Clinical Medicine*, **131**(3), 207–214.

Sepa-Kishi, D. M., Sotoudeh-Nia, Y., Iqbal, A., Bikopoulos, G., & Ceddia, R. B. (2017). Cold acclimation causes fiber type-specific responses in glucose and fat metabolism in rat skeletal muscles. *Scientific Reports*, **7**(1), 15430.

Shadrin, I. Y., Khodabukus, A., & Bursac, N. (2016). Striated muscle function, regeneration, and repair. *Cellular and Molecular Life Sciences*, **73**(22), 4175–4202.

Shibaguchi, T., Hoshi, M., Yoshihara, T., Naito, X. H., Goto, K., Yoshioka, T., & Sugiura, T. (2019). Impact of different temperature stimuli on the expression of myosin heavy chain isoforms during recovery from bupivacaine-induced muscle injury in rats. *Journal of Applied Physiology*, **127**(1), 178–189.

Shibaguchi, T., Sugiura, T., Fujitsu, T., Nomura, T., Yoshihara, T., Naito, H., Yoshioka, T., Ogura, A., & Ohira, Y. (2016). Effects of icing or heat stress on the induction of fibrosis and /or regeneration of injured rat soleus muscle. *The Journal of Physiological Sciences*, **66**(4), 345–357.
Shiota, S., Okada, T., Naitoh, H., Ochi, R., & Fukuchi, Y. (2004). Hypoxia and hypercapnia affect contractile and histological properties of rat diaphragm and hind limb muscles. *Pathophysiology*, **11**(1), 23–30.
Siques, P., Brito, J., Flores, K., Ordenes, S., Arriaza, K., Pena, E., León-Velarde, F., de Pablo, Á. L. L., Gonzalez, M. C., & Arribas, S. (2018). Long-term chronic intermittent hypobaric hypoxia induces glucose transporter (GLUT4) translocation through AMP-activated protein kinase (AMPK) in the soleus muscle in lean rats. *Frontiers in Physiology*, **9**, 799.
Souza, J. D., & Gottfried, C. (2013). Muscle injury: Review of experimental models. *Journal of Electromyography and Kinesiology*, **23**(6), 1253–1260.
Tidball, J. G. (2011). Mechanisms of muscle injury, repair, and regeneration. *Comprehensive Physiology*, **1**(4), 2029–2062.
Tiidus, P. M. (2008). *Skeletal Muscle Damage and Repaired* (M. P. Tiidus Ed.). Human Kinetics.
Toumi, H., F'guyer, S., & Best, T. M. (2006). The role of neutrophils in injury and repair following muscle stretch. *Journal of Anatomy*, **208**(4), 459–470.
Turner, N. J., & Badylak, S. F. (2012). Regeneration of skeletal muscle. *Cell and Tissue Research*, **347**(3), 759–774.
Valle, X., Malliaropoulos, N., Párraga Botero, J. D., Bikos, G., Pruna, R., Mónaco, M., & Maffulli, N. (2018). Hamstring and other thigh injuries in children and young athletes. *Scandinavian Journal of Medicine & Science in Sports*, **28**(12), 2630–2637.
Vieira Ramos, G., Pinheiro, C. M., Messa, S. P., Delfino, G. B., Marqueti, R. D. C., Salvini, T. D. F., & Durigan, J. L. Q. (2016). Cryotherapy reduces inflammatory response without altering muscle regeneration process and extracellular matrix remodeling of rat muscle. *Scientific Reports*, **6**, 18525.
Viscor, G., Corominas, J., & Carceller, A. (2023). Nutrition and hydration for high-altitude alpinism: A narrative review. *International Journal of Environmental Research and Public Health*, **20**(4), 3186.
Viscor, G., Torrella, J., Fouces, V., & Palomeque, J. (1992). Skeletal muscle capillarization and fiber types in urban and homing pigeons (Columba livia). *Comparative Biochemistry and Physiology-Comparative Physiology*, **101**(4), 751–757.
Viscor, G., Torrella, J. R., Corral, L., Ricart, A., Javierre, C., Pages, T., & Ventura, J. L. (2018). Physiological and biological responses to short-term intermittent hypobaric hypoxia exposure: From sports and mountain medicine to new biomedical applications. *Frontiers in Physiology*, **9**, 814
Warren, G. L., Lowe, D. A., & Armstrong, R. B. (1999). Measurement tools used in the study of eccentric contraction-induced injury. *Sports Medicine (Auckland, N.Z.)*, **27**(1), 43–59.
Wei, X., Luo, L., & Chen, J. (2019). Roles of mTOR signaling in tissue regeneration. *Cells*, **8**(9), 1075.
West, J. B., Schoene, R. B., Luks, A. M., & Milledge, J. S. (2012). *High Altitude Medicine and Physiology* (5th edn). CRC Press.
Whalen, R. G., Harris, J. B., Butler-Browne, G. S., & Sesodia, S. (1990). Expression of myosin isoforms during notexin-induced regeneration of rat soleus muscles. *Developmental Biology*, **141**(1), 24–40.
Zhang, P., Liang, X., Shan, T., Jiang, Q., Deng, C., Zheng, R., & Kuang, S. (2015). mTOR is necessary for proper satellite cell activity and skeletal muscle regeneration. *Biochemical and Biophysical Research Communications*, **463**(1–2), 102–108.

Additional information

Data availability statement

The data that support the findings of this study are available from the corresponding author, G.V., upon reasonable request.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author contributions

Conceptualization and funding acquisition: T.P., G.V. and J.R.T.; Supervision: G.V. and J.R.T. Data curation: G.S. and J.R.T; Formal analysis: G.S., G.V. and J.R.T. Writing – original draft: G.S.; Writing – review & editing: G.S., G.V. and J.R.T. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualified for authorship and all those who qualified for authorship are listed as authors.

Funding

This work was supported by the Spanish Ministry of Economy, Industry and Competitiveness (DEP2013-48334-C2-1-P) and the University of Barcelona (graduate fellowship APIF 2016—2017 to G.S.).

Acknowledgements

The authors are grateful for the contribution of Santiago Ruvira, Ignacio Cabrera, Silvia Lara, Marc Comas, Deisy C. Ortiz, Cristian A. Guzmán and Ana M. Ortega in some laboratory tasks.

Keywords

AKT, AMPK, mTOR, muscle function, muscle histology, muscle injury, regeneration

Supporting information

Additional supporting information can be found online in the Supporting Information section at the end of the HTML view of the article. Supporting information files available:

Peer Review History